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METHOD FOR BIO-ASSAY OF HORMONES OF ADRENAL CORTEX WHICH INFLUENCE DEPOSITION OF GLYCOGEN IN THE LIVER

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Not only does an extract of the adrenal cortex influence renal function and the distribution and excretion of electrolytes but, in addition, it has a marked effect on carbohydrate metabolism (1). No single method can be used to determine the potency of an extract of the adrenal cortex in regard to both renal function and carbohydrate metabolism. For bio-assay of the influence on the kidney the determination of changes of the concentration of urea in the blood of the adrenalectomized dog has been shown to be satisfactory (1, 2) but no procedure for the bio-assay of the potency of an extract of the adrenal cortex in respect to carbohydrate metabolism has been widely accepted. Since such a method would be of great assistance, particularly for standardization of preparations used in clinical medicine, we have carried out an investigation directed toward this objective. Changes of the rate of gluconeogenesis (3), depression of the respiratory quotient (4, 5, 6), an increase of the concentration of glucose in the blood (5, 7), inhibition of the utilization of glucose (2, 4), an antagonism to insulin (1, 8) and deposition of glycogen in the liver (5,7) are all manifestations of the influence of some of the hormones of the adrenal cortex on carbohydrate metabolism. Although many observations on these effects have been recorded for several years, and although Britton and Silvette (7) and later Long and his associates (5), have investigated the influence of extracts of the adrenal cortex on the deposition of glycogen in the liver, the use of a method of bio-assay for the potency of preparations of the adrenal cortex in respect to the effect on carbohydrate metabolism has not been reported.

There are four considerations which indicate that deposition of glycogen in the liver is the most suitable criterion for the basis of a method for the bio-assay of the hormones of the adrenal cortex which influence carbohydrate metabolism: *a*) After 24 hours of fasting the glycogen in the liver of an adrenalectomized rat has been found without exception to be very low (less than 0.1 per cent). This insures a remarkably constant base line and eliminates false, positive results. *b*) The deposition of glycogen is a positive function of the amount of extract injected within the reasonably wide limits which have been adopted. *c*) The influence of the hormones of the adrenal cortex on the deposition of glycogen is highly specific. Only those compounds which have

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an atom of oxygen on carbon 11 exert any influence (8, 9). *d*) The determination of glycogen in the liver can be carried out without the use of unusual or elaborate apparatus.

The adrenalectomized rat was chosen as the assay animal for reasons of economy and convenience. Male rats were used because they were more readily available at this laboratory and because they do not have the complications associated with the estrous cycle. The range in weight of 140 to 160 gm. was chosen after some difficulty had been encountered in an attempt to use smaller animals. In some of the experiments somewhat heavier animals were used but this did not offer any definite advantage and did not alter the results appreciably. A period of one day of fasting preceding the day on which the assay was made was found to be sufficient to reduce the liver glycogen practically to zero. This procedure was tolerated well by the animals.

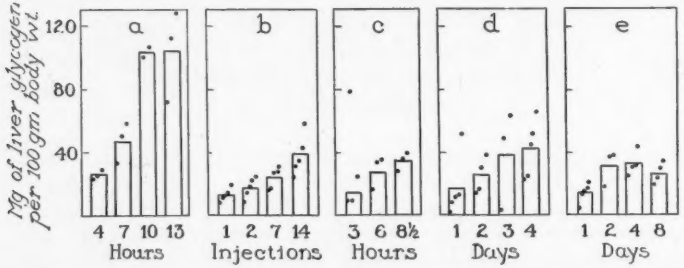

Fig. 1*a*. Rate of deposition of glycogen in the liver caused by the administration of large amounts of adrenal cortical extract. Heights of the bars represent the averages for the groups. Dots indicate the individual values. These animals were used on the second day after adrenalectomy. One cc. per hour of a potent extract was given for the period indicated for each group.

b. Relation of frequency of administration to the apparent potency of an extract. Animals in the first group were given one injection of 1.4 cc. of a potent extract at the beginning of the assay period, those in the second group were given 0.7 cc. in 2 injections at intervals of 3.5 hours, those in the third group 0.2 cc. per hour for 7 hours and those in the fourth group 0.1 cc. each half-hour for 7 hours.

c. Deposition of glycogen in the liver after a single large injection of extract. These animals were used on the second day after adrenalectomy and were given one injection of 5 cc. of a potent extract and then killed at the intervals indicated. They were given tap water in place of saline solution throughout and an injection of potent extract on the first day after adrenalectomy.

d and *e*. Effect of the length of the period after adrenalectomy on the response to a given dose of extract. These animals were used on the days indicated after adrenalectomy. They were all given 0.2 cc. per hour of a potent extract.

There now remained the choice of the time during which the cortical extracts should be allowed to act, the frequency with which they should be administered and the number of days after adrenalectomy that the animals should be allowed to live before being used.

A study of the rate of deposition of glycogen in the liver during the administration of large doses of extracts (fig. 1*a*) showed that reasonable amounts were formed within a period of 7 hours. More glycogen was deposited after 10 or more hours but these longer periods entailed not only inconvenience but also the possibility that the amount of glycogen deposited would be determined not by the amount of extract given but by the potentialities of the animal.

A study of the deposition of glycogen caused by a given dose of extract variously subdivided (fig. 1*b*) indicated that within the limits of the experiment the effect of the extract increased with the increasing frequency of administration. However, as a matter of practical convenience, hourly administration was adopted. It is probable that the effect of a single injection may have decreased significantly by the time the

livers were removed for analysis. But this in itself precludes the possibility of the use of single or infrequent injections since approximately 7 hours must be allowed for the development of an adequate response at the higher levels of dosage. The data of figure 1c indicate that large doses of extract may exert a prolonged effect, and this may be important in the clinical use of extracts, in which convenience may be of more importance than the loss of some of the effectiveness.

The fourth day after adrenalectomy was finally chosen as the day on which the animals were to be used for assay, since at this time the animals were apparently able to respond with the largest deposition of glycogen for a given dose of extract (fig. 1*d* and *e*).

To summarize the method finally adopted: male rats weighing 140 to 160 gm. are adrenalectomized by the usual lumbar route and given 1 per cent saline solution and colony diet ad libitum (Purina fox chow, Purina dog chow or Alber's friskies). During

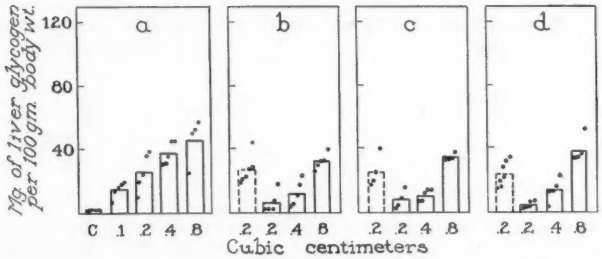

Fig. 2*a*. VARIATIONS OF THE AMOUNT OF GLYCOGEN DEPOSITED BY DIFFERENT AMOUNTS OF EXTRACT. Animals in group C were given 0.8 cc. per hour of the same extract used on the other groups shown in the figure after it had been thoroughly extracted with chloroform. Volumes indicated were the hourly doses for the respective groups.

b, c and *d*. DEPOSITION OF GLYCOGEN IN THE LIVER caused by various volumes of samples of adrenal cortical extracts prepared by commercial manufacturers. Control groups shown at the left in each figure (indicated by bars drawn with broken lines) were given extract from the same preparation as those in *a*. Volumes indicated were the hourly doses for the respective groups.

the third day after adrenalectomy the food is removed. Injections of the extracts to be assayed are given subcutaneously at hourly intervals beginning the morning of the fourth day after adrenalectomy. Between 1 and 2 hours after the last injection of adrenal extract the animals are anesthetized with an injection, given intraperitoneally, of 9 mg. of sodium amytal per 100 gm. of body weight. The entire livers are dissected out and dropped into a hot 30 per cent solution of potassium hydroxide, the volume of which is approximately 2 cc. for each gram of liver. They are then analyzed for their total glycogen content by the method of Good, Kramer and Somogyi (10). The glycogen is calculated as milligrams per 100 gm. of body weight. This procedure was found to give slightly more uniform results than the calculation of glycogen expressed as percentage of weight of liver and obviated the necessity of determining accurately the weight of the liver.

With the exceptions individually noted, this procedure was followed exactly in all of the experiments reported in this paper. When various dosages of a single sample of extract prepared in this laboratory were used, the amount of glycogen deposited in the liver per 100 gm. of body weight was found to be a positive function of the dosage in the range studied (fig. 2*a*). Extraction of the aqueous solution of the cortical hormones with chloroform was found to remove all influence on the deposition of glycogen in the liver.

After it had been shown that deposition of glycogen in the liver could be used as one measure of the potency of an extract of the adrenal cortex it became a matter of some interest to compare extracts of the adrenal cortex prepared by pharmaceutical manufacturing companies. The results given by three such samples are shown in figure 2*b*, *c* and *d*. All of these samples were less potent on the basis of this method of assay than the product made in this laboratory, which was used as a control in each case. This held true, not only when the potency was calculated on a volume basis as shown, but also when it was calculated on the basis of the weight of gland used in the preparation of each of the extracts.

Because of the small number of animals used in each group there is some possibility of error in the assay of these extracts, but the consistency of the increase of the average amount of glycogen deposited with increasing dosage in each case indicates that this possibility is rather small. The determination of exact quantitative differences cannot be made with a small number of rats but the use of a larger number of

TABLE 1. DIFFERENTIATION OF A ONE-FOLD DIFFERENCE IN THE AMOUNT OF EXTRACT GIVEN

Hourly Dose of Extract, cc.	Liver Glycogen, mg. per 100 gm. Body Weight											Mean	Difference	Standard Error
0.2	8.7	9.9	9.9	10.6	16.7	17.8	18.2	19.5	19.8	21.2	32.0	16.7	18.0	±4.5
0.4	18.8	20.5	23.0	27.1	29.1	31.8	36.5	40.7	41.1	55.7	57.8	34.7		

animals in each group makes the detection of a one-fold difference in potency reasonably certain (table 1).

COMMENT

The observation that extracts of the adrenal cortex are more potent when given at frequent intervals supports a similar observation made by Britton and Silvette (7). Since a one-fold difference in the amounts of extract administered produced a difference in the average amount of glycogen deposited which was approximately 4 times the standard error (table 1), it would seem probable that this method could be adapted to detect much smaller differences in potency if the number of animals used was increased. It should be pointed out that for the comparison of potency, the most satisfactory results are obtained when the amount of glycogen deposited in the liver is between 15 and 75 mg. for each 100 gm. of body weight.

This method for the bio-assay of extracts of the adrenal cortex is not based on the determination of the minimal amount of extract which will produce an increase of glycogen in the livers of treated rats when compared with the corresponding weights of glycogen in untreated rats. The most satisfactory basis for the bio-assay was found to be the determination of the amount of glycogen deposited in the liver per 100 gm. of body weight after the administration of increasing volumes of a standard preparation and the simultaneous determination of the response to the injection of the same volumes of a solution of unknown potency. Comparison of these two results indicates the physiologic activity of the unknown solution in terms of the standard preparation.

The concentrations of glycogen per 100 gm. of body weight in the livers of six rats which were treated precisely as described but which did not receive any extract were 0.10, 0.18, 0.26, 0.26, 0.30 and 0.44 mg. In the livers of similar rats treated with

0.8 cc. of extract of the adrenal cortex the glycogen in the liver per 100 gm. of body weight was increased from one hundred to four hundred times these amounts. The determination of glycogen was made without correction for nonfermentable reducing substances since the low order of the total reducing substances in the untreated rats indicated that the correction would be without significance.

The use of young rats of less than 140 to 160 gm. of body weight was not satisfactory. The variations in the amounts of glycogen deposited in the livers of these rats indicate the necessity of using older animals. No satisfactory explanation can be given at the present time for the variability encountered in the younger rats.

SUMMARY

A method for the bio-assay of extracts of the adrenal cortex is described. The criterion used to determine the potency is the amount of glycogen deposited in the liver of a fasting adrenalectomized rat. With groups of 5 or 6 rats differences of one-fold can be detected, but for greater accuracy larger groups are recommended.

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EFFECT OF SYNTHETIC DESOXYCORTICOSTERONE ACETATE THERAPY ON PLASMA VOLUME AND ELECTROLYTE BALANCE IN NORMAL DOGS[1]

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EARLIER experiments indicated that the intramuscular injection of synthetic desoxycorticosterone acetate in oil rapidly induced striking dilution of the blood in adrenalectomized dogs (1). Previous studies in normal dogs (2) indicated that long-continued administration of excessive quantities of desoxycorticosterone acetate was followed by undesirable consequences. Direct plasma volume measurements were not reported in these experiments.

In the present study an attempt has been made to follow the changes in plasma volume which occur during a relatively short period of therapy with desoxycorticosterone acetate. The efficacy of intramuscularly and sublingually administered hormone has been compared with and without supplementary sodium chloride medication.

METHODS

Normal male dogs (approximately 10 to 15 kg. in weight) were kept in metabolism cages and fed a constant diet consisting of 350 gm. of raw, ground beef and 50 cc. of irradiated evaporated milk to which was added 25 cc. of 10 per cent sodium chloride solution. Sufficient water was given to make a total fluid intake of 475 cc. daily. All fluids were administered by stomach tube. Twenty-four-hour urine specimens were collected under toluol, measured, and a 4-ounce aliquot saved for chemical analysis. Completeness of urine collections was assured by catheterization of the animals each morning. This was easily accomplished with the use of a No. 7 French silk catheter. The cage bottoms were washed at this time with 100 cc. of water and these washings were added to the 24-hour-urine sample. Following this, the cages were cleaned and washed out with hot water. Blood samples were removed at appropriate intervals from the external jugular vein with minimal stasis. Plasma volume measurements were made according to the technique of Gregersen and Gibson (3, 4) using the dye, T-1824. Duplicate samples of blood for serum were withdrawn at intervals of 15 minutes during a period of one hour after the administration of the dye. The concentration of the dye in the samples of serum was determined by means of the Evelyn photoelectric colorimeter (5, 6). In order to reduce the amount of blood needed for the determination and to minimize error due to hemolysis, relatively large amounts of the dye were administered. The colorimeter values on serum were read at a depth of 0.1 cc. For this purpose a long plunger (1.9 cm.) was used.

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The following blood analyses were made: hematocrit (7), serum sodium (8), chloride (9), potassium (10, 11), carbon dioxide combining power (12, 13) and total protein by measurement of refractive index (14). Twenty-four-hour urine samples were measured and analyzed for sodium (8) and chloride (9).

Desoxycorticosterone acetate dissolved in sesame oil (1 cc. = 5 mg.) was administered intramuscularly and a solution of desoxycorticosterone acetate in propylene glycol (1 cc. = 8.5 mg.) was administered sublingually by dropper. The propylene glycol solution was administered in 3 divided doses of 24 drops each, at intervals of 4 hours, a total dose of 15 mg. daily being given. The animals were prevented from swallowing the solution by keeping their jaws tightly closed for a period of 2 minutes following administration of the hormone by dropper.

In one group of experiments the daily sodium chloride intake was increased to a total of 7.5 gm. Under these conditions it was necessary to give the additional sodium chloride in divided doses, well diluted with water to avoid gastro-intestinal irritation and vomiting.

OBSERVATIONS

The effect of the daily intramuscular administration of 5 mg. of desoxycorticosterone acetate on plasma volume, hematocrit values and electrolyte balance of 3

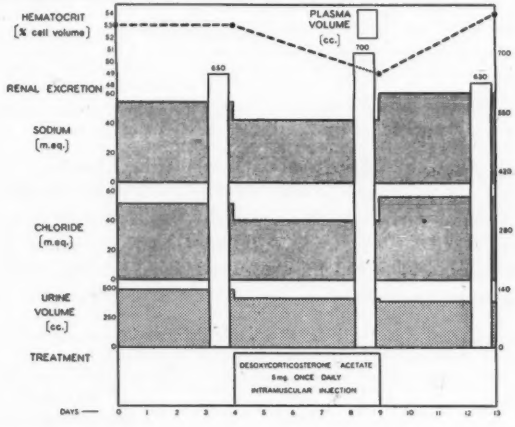

Fig. 1. Effect of intramuscular injections of desoxycorticosterone acetate on electrolyte balance (average values—3 normal dogs).

normal dogs is shown in figure 1. In this experiment all plasma volume values represent dye measurement. The increase in plasma volume resulting from therapy was accompanied by a fall in hematocrit value from 53 to 49 per cent, average (table 1). During the treatment period there was also a decrease in the renal excretion of sodium chloride amounting to 12 m. eq. of sodium and 11 m. eq. of chloride daily. Withdrawal of desoxycorticosterone acetate therapy was followed by an increase in renal excretion of sodium and chloride level above that observed during the control period.

The serum concentration of sodium and chloride also increased during therapy, rising from an average value of 142 m.eq. per liter for sodium and 112 m.eq. per liter for chloride prior to therapy to an average value of 153 for sodium and 120 for chloride at the end of the 5-day period of treatment. The increase in concentration of the ions

in the serum is of particular interest in that it occurred in conjunction with an increase in plasma volume. Thus, a relatively great increase in the total quantity of sodium and chloride in the serum occurred during the period of therapy.

The effect of the sublingual administration of a total of 15 mg. of desoxycorticosterone acetate, in 3 divided daily doses of 5 mg. each is shown in figure 2. The changes

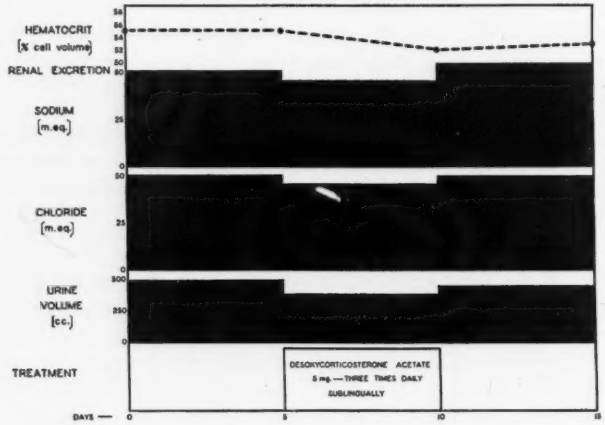

Fig. 2. Effect of sublingual administration of desoxycorticosterone acetate on electrolyte balance (average values—3 normal dogs).

in hematocrit values and electrolyte balance are similar in character to those produced by 5 mg. of desoxycorticosterone acetate administered intramuscularly, but are of considerably less magnitude. There was decidedly less increase in plasma volume, as reflected by the fall in hematocrit values (table 1), than was noted in the previous experiment in which hormone was injected intramuscularly in a much smaller dose. The retention of sodium and chloride during the treatment period with sublingual

Table 1. Effect of desoxycorticosterone acetate treatment and supplementary sodium chloride medication of hematocrit values in normal dogs

Treatment	Hematocrit (Per cent Packed Red Blood Cell Volume)		Calculated Increase in Plasma Volume, cc.
	Prior to therapy	Fifth day of therapy	
Desoxycorticosterone acetate in oil, 5 mg.; single daily intramuscular injection	52.9	48.9	50
Desoxycorticosterone acetate in oil, 5 mg.; single daily intramuscular injection; 5 gm. of sodium chloride added.	52.6	50.1	32
Desoxycorticosterone acetate in propylene glycol, 15 mg. daily; administered sublingually in divided doses	54.5	52.1	30
Desoxycorticosterone acetate in propylene glycol, 15 mg. daily; administered sublingually in divided doses; 5 gm. of sodium chloride added	51.9	49.7	28
5 gm. of sodium chloride added	52.9	51.9	12

hormone (15 mg. daily) amounted to 7 and 6 m.eq. per day, respectively, for the 5-day period.

The effect of the daily administration of 5 gm. of sodium chloride on hematocrit value and plasma volume is shown in table 1. During a period of 5 days of treatment a slight fall in hematocrit value occurred. This was equivalent to an increase in plasma volume of approximately 12.5 cc.

The simultaneous administration of desoxycorticosterone acetate and 5 gm. of sodium chloride did not enhance the increase in plasma volume produced by desoxycorticosterone acetate therapy alone. In fact, the increase in plasma volume produced by hormone and sodium chloride treatment was actually slightly less than that following hormone therapy alone (table 1).

DISCUSSION

It was evident from these experiments that the daily intramuscular injection of 5 mg. of desoxycorticosterone acetate induced a prompt and appreciable increase in plasma volume in normal dogs. The sublingual administration of much larger quantities of hormone was less effective in this respect. It was of interest to note that the administration of 5 gm. of additional sodium chloride daily did not augment the effect of hormone treatment on plasma volume. One explanation of this may be that the increased renal excretion of sodium and chloride during the period of increased sodium chloride administration required the utilization of a large proportion of the daily fluid intake. Hence, under conditions of constant fluid intake, less water was available for increasing plasma volume. This hypothesis is substantiated by the increase in urine volume which occurred during the period of therapy with additional sodium chloride.

SUMMARY AND CONCLUSIONS

A single daily intramuscular injection of 5 mg. of synthetic desoxycorticosterone acetate in oil for 5 days resulted in an appreciable increase in plasma volume in normal male dogs. The sublingual administration of 15 mg. of hormone daily (5 mg. 3 times daily) was less effective in this respect. Supplementary sodium chloride medication did not enhance the effect of synthetic hormone on plasma volume.

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INACTIVATION OF DESOXYCORTICOSTERONE ACETATE

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IN A PREVIOUS report (1), a method for the study of inactivation of estrone and testosterone propionate was described, and has been employed to study the inactivation of insulin (2). Burrill et al. (3) utilized this technique to study the inactivation of desoxycorticosterone. Eversole et al. obtained contradictory results by a similar method (4) and by the use of adrenal cortical transplants (5).

The present experiments were devised to investigate these contradictions and were an attempt to determine the site of inactivation of desoxycorticosterone in the body.

METHOD

Male white rats 100 days old[1] were kept in individual cages which contained a food cup and 3 accurately graduated 90 cc. inverted drinking bottles; one was filled with distilled water, one with a 3 per cent solution of sodium chloride, and one with a 40 per cent solution of sucrose. The food cup contained a diet (6) consisting of: saltless butter, 50 gm.; calcium carbonate, 15 gm.; graham flour, 725 gm.; casein, 100 gm.; skim milk powder, 100 gm.[2] The position of the bottles in the cage remained the same throughout the experiment. Fluid intake from each bottle was recorded daily. When the saline intake was minimal, the adrenals were removed. Our technique for adrenalectomy was similar to Richter's (6) but our survival rates did not approximate 100 per cent as in his series. Some rats survived as long as 15 to 20 days following adrenalectomy with access to sodium chloride. This is similar to the experience of Gaunt et al. (7).

The possible rôle of the liver or spleen in the inactivation of desoxycorticosterone was investigated by the implantation of 3 pellets of desoxycorticosterone acetate[3] subcutaneously in the control series of 5 rats, and intra-splenically in another series of 5 rats. These pellets were made of crystalline desoxycorticosterone acetate in a punch press previously described (8). An attempt to use 30-day-old male rats was unsuccessful, since the spleen is too small to accommodate 3 pellets of this weight.

RESULTS

The effect of adrenalectomy and treatment with desoxycorticosterone pellets upon salt, sucrose and water appetite has been described (9). The present report is concerned with changes in the salt appetite.

The average daily sodium chloride intake for 5 control 100-day-old male rats

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[1] Albino rats supplied by The Albino Rat Farms, Red Bank, N. J.

[2] Diet upon analysis contained 0.033 mg. chloride per 100 gm. of food mixture.

[3] Dr. Robert Mautner of Ciba Pharmaceutical Company kindly supplied desoxycorticosterone acetate (Percorten) for these experiments.

increased from 1.3 cc. for a 10-day period immediately before adrenalectomy to 9.1 cc. for a 10-day post-operative period.

Three pellets of desoxycorticosterone were then inserted subcutaneously into the dorsal space of the back. The original total weight of the 3 pellets was 23.2 to 25.4 mg. They remained *in situ* for 16 days. The average daily absorption was 0.38 mg. Under treatment the average daily sodium chloride intake of the adrenalectomized rats decreased from 9.1 to 2.1 cc.

In a second group of five 100-day-old male rats, the average daily intake of sodium

TABLE 1. AVERAGE DAILY NaCl INTAKE BEFORE AND AFTER ADRENALECTOMY AND DURING TREATMENT WITH DESOXYCORTICOSTERONE PELLETS

Rat No.	Sodium Chloride Intake cc. of 3% Solution			Absorption from Desoxycorticosterone Pellets			
	10 days before	A 10-day period after	First 10 days of treatment	Wt. of 3 pellets in mg.	Days *in situ*	Total absorption in mg.	Average daily absorption in mg.
Subcutaneous pellets							
5	1.3	11.9	2.2	25.4	16	6.7	0.42
7	1.3	10.8	2.8	25.2	16	6.5	0.41
10	1.0	9.1	1.9	26.0	16	6.6	0.41
11	0.8	6.1	1.8	23.1	16	3.8	0.24
13	1.9	7.5	1.6	23.2	16	7.0	0.44
Av.	1.3	9.1	2.1				
Intrasplenic pellets							
3	0.9	7.2	1.9	24.7	10	3.0	0.30
15	2.3	7.4	2.9	23.3	10	3.8	0.38
18	1.0	10.7	3.4	24.3	10	3.1	0.31
19	1.8	10.7	1.9	24.1	10	4.5	0.45
20	3.8	7.9	3.7	22.2	14	5.8	0.41
Av.	2.0	8.8	2.8				0.38

chloride increased from 2.0 cc. for a 10-day period immediately before adrenalectomy to 8.8 cc. for a 10-day postoperative period.

Three pellets of desoxycorticosterone were then inserted intrasplenically by means of a large-bore needle and stylet. The original total weight of the 3 pellets was 22.2 to 24.7 mg. The pellets remained *in situ* for 10 to 14 days. The average daily absorption was 0.38 mg. Under treatment with the hormone in the splenic site, the average daily sodium chloride intake of adrenalectomized rats decreased from 8.8 cc. to 2.8 cc., a decrease comparable to the effect obtained by subcutaneous pellets.

The rats in both groups were autopsied at the termination of the experiment. The pellets in the second group were found in the splenic pulp without perisplenic adhesions. A much larger group was utilized originally, but a number died following the operation for splenic implantation of pellets, and others were discarded because of perisplenic adhesions; in some instances pellets had extruded from the splenic pulp, and absorption had occurred by way of the peritoneum.

All intact rats did not reach a low sodium chloride intake prior to operation. Rat 6, which was observed for more than 40 days, had an average daily sodium chloride intake of 5.5 cc. However, each rat served as his own control, since all rats that had

a low salt intake before adrenalectomy, greatly increased the intake following operation, and then returned to the low intake following treatment. All of the rats which survived adrenalectomy with access to sodium chloride, did not survive for a long period. A number died 15 to 20 days following adrenalectomy.

DISCUSSION

In these experiments, a control series of rats, whose originally low intake of sodium chloride increased after adrenalectomy, showed a reversal of sodium chloride intake to the originally low levels following subcutaneous implantation of desoxycorticosterone. In an attempt to decide if the liver or spleen is responsible for the inactivation of desoxycorticosterone in the body, a similar amount of hormone was implanted directly into the spleen. Following absorption from this site, the desoxycorticosterone passes through the splenic vein to the liver, without access to other venous or lymphatic drainage areas. The same effect was obtained in this group; namely, a reversal of the increased sodium chloride intake following adrenalectomy to the original low level.

Burrill et al. (3), who investigated this same problem, inserted pellets of similar weight into the mesentery of the intestine. Their criterion of activity, was maintenance of life following adrenalectomy in rats receiving only water. They concluded that, in this dosage, inactivation took place in the liver; in the case of much larger dosage, however, they felt inactivation of the hormone was incomplete. It must be remembered too, that absorption from the intestinal mesentery allows lymphatic absorption as well as access to the portal vein, and that these lymphatics do not pass through the liver but empty into the thoracic duct.

Eversole et al. (5) transplanted adrenal cortical tissue to the intestinal mesentery and found no impairment of the life-maintenance factor by this hepatic portal drainage. In another experiment, pellets of desoxycorticosterone and cholesterol were implanted into the spleen of young adrenalectomized rats (4). Similar pellets were implanted in control animals in the kidney, subcutaneously, and intraperitoneally. They found no significant differences in growth and survival times between rats with pellets in the spleen and those with pellets located elsewhere.

In a large preliminary series of animals we were unable to obtain satisfactory uniform mortality rates following adrenalectomy in any but 30-day-old male rats which had access to water alone. This confirms the experiences of others (10) who found a marked colony difference in the survival of adrenalectomied adult rats. They reported that 64 per cent of one adult colony survived adrenalectomy as compared to 12.5 per cent in another, and that the 30-day-old rats of both colonies showed more comparable high mortality rates. In another report (7) these workers found an appreciably increased percentage of survival and prolongation of life in 30-day-old and adult rats which received sodium chloride following adrenalectomy. North (11) who has performed more than 15,000 adrenalectomies in rats for standardization of adrenal cortical extract, finds a definite colony variation in the percentage of survivals following adrenalectomy. The most satisfactory uniformity of results is obtained with 30-day-old male rats of certain dealers; 90 to 95 of 100 will die within 10 days following operation. The results are unpredictable when adult male and female rats from these dealers as well as rats from other sources are used.

Considering the lack of uniformity in survival following adrenalectomy under the same conditions in the same hands, and that survival depends upon the colony of rats, their age and sex, it seems hazardous to draw conclusions based on the sur-

vival of animals in a small series, unless 30-day-old males from a controlled colony are used.

It was found that the salt appetite followed a definite pattern in a given rat, before and after adrenalectomy and after desoxycorticosterone treatment. Although all of these rats had access to water and sucrose, these intakes were not included in this report because these changes did not show a significant pattern (9).

CONCLUSIONS

Ten adrenalectomized 100-day-old male albino rats were given access to 3 per cent sodium chloride solution, distilled water, 40 per cent sucrose solution and salt-less stock diet.

2. Following adrenalectomy there was a marked increase in the intake of sodium chloride solution.

3. Absorption of an average of 0.38 mg. per day of desoxycorticosterone acetate from 3 pellets placed subcutaneously, decreased the sodium chloride intake to normal levels.

The same type of pellets placed in the spleen, caused the same decrease in sodium chloride intake.

4. From these experiments there is no evidence that desoxycorticosterone is inactivated by the liver or spleen.

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INFLUENCE OF DIETHYLSTILBESTROL UPON THE ADRENAL CORTEX OF THE GUINEA PIG

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THAT THERE IS an intimate relation between gonadal and adrenal cortical function seems well established. Andersen and Kennedy (1) showed that the adrenal glands vary in size and histology at different stages in the estrous cycle, reaching their maximum development during estrus. Zuckerman, Bourne and Lewes (2) produced cyclical changes in the adrenal glands of spayed rats over 5-day periods by the daily injection of a threshold dose of estrone. Here, too, the adrenals were found to be significantly hypertrophied at the time of estrus. That this adrenal hypertrophy occurs at the usual time of greatest estrogen concentration in the blood is consistent with the majority of data, discussed below, upon the effect of estrogen treatment upon the adrenal glands.

Conversely, Corey and Britton (3) have shown that the normal estrous cycle is dependent upon a functioning adrenal cortex. Long and Zuckerman (4) advance the view that the adrenal cortex is responsible, in part at least, for the cyclical changes occurring in the uterus of spayed female monkeys. Cramer and Horning (5) have shown *a*), that estrogen will often prevent the mortality occurring after adrenalectomy, *b*) that adrenalectomy lessens the ill effects of heavy estrogen treatment on body weight and growth, and *c*) that it ameliorates the degenerative changes that estrogen produces in the gonads. From their work they conclude that there is a synergic relationship between ovarian estrogenic hormone and adrenal hormone.

Hoffmann (6) presents data to the effect that the adrenal cortex of domestic animals contains a gonadotropin in addition to the cortin factor, and that there is an inverse relation between the cortin content and the cortical-gonadotropin content. Howard and Grollman (7), on the other hand, conclude from their work on rats that there is no specific sex-maintaining factor.

Further data upon the problem of gonad-adrenal relationships are found in experiments upon the effects of gonadectomy or sex hormone administration upon the adrenal glands. Andersen and Kennedy (8) review the early work on the effect of gonadectomy upon the adrenal glands in different species of animals. Their own work indicated that results in both male and female rats were dependent on the age at which the gonads were removed and on the length of time elapsing between the operation and autopsy of the animals. Depending on these two factors, hypertrophy, atrophy and no change of the adrenals resulted from removal of the gonads. However, later work by Winter and Emery (9), Freudenberger and Howard (10), Zeckwer (11), Blumenfeld (12), Hall (13), and Pinto (14), all on rats, has tended to show that gonadectomy in the male results in hypertrophy and in the female in atrophy of the adrenals. Hashimoto (15) gives us a clue to the reasons for diversity in the conclusions drawn from experiments upon the effect of ovariectomy upon the adrenal glands. In a large number of experiments upon immature rats he found that removal of the ovaries causes an initial hypertrophy of the adrenals evident at 5 to 10 weeks. This was followed by a cessation of growth observed at 10 to 13 weeks, later followed by atrophy for 4 weeks after which there was no further reduction in the weight of the adrenals.

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Blumenfeld (12) reports that atrophy resulting from ovariectomy first involves a decrease in volume of the cells of the zona fasciculata in 3-month-old rats; later the entire cortex shrinks. Hall (13) claims only a slight shrinkage of the zona glomerulosa and slight changes in the zona reticularis of ovariectomized females. Pinto (14) says that the decrease in adrenal weight in ovariectomized females is due to reduction in the thickness of the cellular columns of the cortex and to the increased vacuolization of the cells. Hall (13) claims that in the castrated male an hypertrophy of the zona fasciculata and of the zona reticularis is responsible for the increase in weight of the gland.

Estrogens administered to rats by several workers were found to produce the following effects on the weights of the adrenal glands.

In the intact male. Hypertrophy reported by Korenchevsky and Dennison (16), by Noble (17, 18, 19), by von Haam, Hammel, Rardin and Schoene (20), by Zondek (21). No change reported by Mellish, Baer and Macias (22).

In the castrated male. No change reported by Korenchevsky and Dennison (16), by Hall (13).

In the intact female. Hypertrophy reported by Ellison and Burch (23), by Noble (17, 18, 19), by Mark and Biskind (24) (using estrone), by von Haam et al. (20), by Pinto (14). On the other hand, Mellish et al. (22), and Mark and Biskind (24) reported that estradiol benzoate had no effect upon the adrenal glands. Zondek (21) states that estrone treatment causes adrenal atrophy in female rats.

In the ovariectomized female. Treatment with estrogen was found to cause adrenal hypertrophy as reported by Leiby (25), by Andersen (26), by Ellison and Burch (23), by Mark and Biskind (24), by Pinto (14). No change was reported by Lauson, Heller and Severinghaus (27), by Hall (13).

Of the above, Mellish et al. (22), Noble (17), and von Haam et al. (20) used diethylstilbestrol as the estrogen. In general, it can be seen that the majority of the workers found hypertrophy in both male and female rats after estrogen treatment.

Korenchevsky and Dennison (16) found that estrone in males produced hypertrophy principally of the zona fasciculata and a reduction in size of the zona glomerulosa, with an increased size of the vacuoles in the cortical cells; Ellison and Burch (23) also observed cellular enlargement in both the zona fasciculata and the zona reticularis in intact and ovariectomized females. Andersen (26) reported that estrone injected into castrated females produced an adrenal histology resembling that of intact rats in estrus which, according to Andersen and Kennedy (1), includes enlargement and increased vacuolization of the cells of the outer two-thirds of the zona fasciculata. Although observing no consistent size changes, Hall (13) found that estrogen injected into gonadectomized rats of both sexes caused cellular atrophy in the zona reticularis and a considerable decrease in the size of the zona glomerulosa, accompanied by a decrease in lipoid content of the entire cortex.

Although the present work is concerned only with the influence of estrogen upon the adrenal cortex, it is important to note the effect of androgens because this helps in the interpretation of our results. Studies along this line made by Hall (13), Mark and Biskind (24), Mazer and Mazer (28), McEwen, Selye and Collip (29), Selye (30), and Selye (31) all failed to show that androgens cause hypertrophy of the adrenal glands of either males or females. On the contrary, it was shown by certain of these authors to cause atrophy. Mazer and Mazer (28) state that androgen treatment of immature and mature female rats caused a reduction in the weight of the adrenal glands to 70 per cent of the weight of those of normal controls.

MATERIALS AND METHODS

A total of 62 male and female guinea pigs were used in this work; 24 were intact controls; 31 were intact and treated with diethylstilbestrol, and 7 were non-treated and gonadectomized. The age of the animals at the start of the experiments was, in *series A*, 2.5 months; *series B*, 1 to 6 months; *series C*, 2.5 months; *series D*, 2 months. The castrated animals of *series E* were gonadectomized for periods of 48 and 125 days. The greater length of time made no significant difference in the results. Many of these animals were littermates, and an attempt was made to establish littermate controls wherever possible.

The intact experimental guinea pigs were given various doses of diethylstil-

TABLE 1. INFLUENCE OF DIETHYLSTILBESTROL TREATMENT UPON ADRENAL OF GUINEA PIG

Group	Sex	Number	Av. Weight of Animals		Av. Weight of Adrenals		% Adrenal Weight Increase Produced by Stilbestrol on Basis of Final Body Weight
			Initial gm.	Final gm.	Actual mg.	Corrected mg.	
Series A, 0.5 mg. diethylstilbestrol 3 times per week, 7 weeks; total amount, 10 mg.							
Control	M	2	442	560	137		
Experimental	M	3	427	479	167	195	42.3
Control	F	3	462	522	108		
Experimental	F	3	380	406	139	179	65.7
Series B, 0.33 mg. diethylstilbestrol 3 times per week, 16 weeks; total amount, 15 mg.							
Control	M	3	464	585	149		
Experimental	M	4	543	588	221	220	47.6
Control	F	4	489	605	155		
Experimental	F	4	452	522	209	242	57.1
Series C, 0.5 mg. diethylstilbestrol 3 times per week, 17 weeks; total amount, 27 mg.							
Control	M	5	433	688	173		
Experimental	M	5	498	595	271	313	80.1
Control	F	4	402	668	186		
Experimental	F	5	389	493	208	283	52.2
Series D, 0.5 mg. diethylstilbestrol 6 times per week, 9.5 weeks; total amount, 27 mg.							
Control	M	3	339	507	162		
Experimental	M	7	338	437	186	216	33.3

bestrol[1] orally as indicated in table 1. Tablets of diethylstilbestrol were placed in small gelatin capsules, and were in this way readily swallowed by the guinea pigs. The animals were watched to see that the capsule and contents had been completely swallowed, and no instance of regurgitation occurred.

All animals were weighed once every week on the same day, 24 hours after food had been removed from the cages. The diet consisted of alfalfa-base rabbit pellets, with fresh cabbage added 3 times weekly.

At the end of the experiment the guinea pigs were killed by a sharp blow on the back of the neck, and several of the organs were removed, weighed, and preserved. Both adrenal glands were removed, cleaned of fat, and weighed fresh on a torsion balance accurate to 0.2 mg. They were then fixed in Bouin's or in Zenker-acetic fluid for 24 hours and stored in 70 per cent alcohol. It was important that the average weight of both adrenals be taken since it was found later by a study of the control glands that in both the male and female the left adrenal is approximately 15 per cent heavier than the right.

For histological study, sections through the middle of one adrenal gland from each animal were cut at 7μ and were stained in Galigher's alum-hematoxylin and eosin or in Mallory's azan. The latter stain was found to give the best differentiation of the cortical zones.

RESULTS

Table 1 shows the influence of diethylstilbestrol upon the weight of the adrenaf glands of guinea pigs. Certain modifying factors need to be considered as sex, age ol the animals, dosage, duration of the experiment and such effect as the diethylstilbestrol may exert upon the weight and general bodily conditions of the experimental animals. Segregation of the groups of animals according to sex and dosage

[1] The diethylstilbestrol was supplied by Eli Lilly & Co., Indianapolis, Ind.

TABLE 2. SUMMARY AVERAGES OF PER CENT WEIGHT INCREASE OF ADRENAL GLANDS PRODUCED BY DIETHYLSTILBESTROL TREATMENT

Series	Male, %	Female, %
A	(3) 42.3	(3) 65.7
B	(4) 47.6	(4) 57.1
C	(5) 80.1	(5) 52.2
D	(7) 33.3	

Average of 19 males, 50.0%; of 12 females, 57.2%.

tends to break up the material into small groups but the concordance of results, enables us to consider the material as a whole. We have assumed that the size of the adrenal glands is roughly proportional to the size of the body. For this reason a correction is made in the weights obtained for the adrenal glands in order to compensate for the smaller size of the animals treated with diethylstilbestrol.

In all groups of experimental animals the adrenal glands were definitely heavier than those of the controls, but this effect is even more marked when the difference in the weight of the control and experimental animals is taken into account. A glance at the table will show that in no case are our conclusions dependent upon this correction.

In order to evaluate the importance of the adrenal medulla as a factor in determining these differences in weight a camera lucida tracing of a transverse section across the center of each adrenal gland was made indicating both the peripheral and the inner boundary of the cortex. These lines were then traced with a planimeter and the total area and the area of the medullary region computed. The area of the latter was found to average 5 per cent of the total area with a range from 4 to 9 per cent. A considerable part of the medulla is made up of large blood vessels that are calculated with it, although not physiologically a part of it. No significant difference was found to exist between the area of the medullary region as determined in the control and animals treated with diethylstilbestrol. This is not surprising because it is to be expected that the relation between estrogens and the adrenal gland would concern the cortex rather than the medulla because of the correlation in the hormones involved.

In any case, the small size of the medullary region makes it a negligible factor because the weight of the adrenals of the animals receiving diethylstilbestrol ranges between 33.3 and 80.1 per cent greater than that of the controls. It seems very clear that treatment of males or females with diethylstilbestrol causes a marked increase in the weight of the adrenal cortex.

Our table would appear to indicate that diethylstilbestrol treatment causes a greater increase in the weight of the adrenal glands in females than in males, but the difference is not great and the number of animals used in the experiment is not large enough to give it significance. In both sexes the increase in weight caused by diethyl-

TABLE 3. INFLUENCE OF CASTRATION UPON WEIGHT OF ADRENAL GLANDS OF GUINEA PIGS

Group	Sex	Number	Average Body Weight gm.	Average Adrenal Weight mg.	Corrected Final Weight of Adrenal Glands mg.	% of Adrenal Weight Produced by Castration
Control	M	5	623	150		
Castrated	M	3	572	130	141	−6.0
Control	F	2	612	140		
Castrated	F	4	515	119	141	0.7

stilbestrol treatment is so universal and so pronounced that there can be no doubt regarding its effect in causing thickening of the adrenal cortex.

Attention was given to the histology of the adrenal cortex in animals treated with diethylstilbestrol as compared with controls. Two criteria were followed, one being the texture of the deep cortex and the other the vacuolization of the zona fasciculata.

The deep cortex shows varying degrees of dissociation of the cells. In some specimens the cords are broken up into glomerular masses, and there often is a pulling apart and shrinkage of the component cells, together with pycnotic changes of the nuclei which, with other changes, give an appearance of degeneration. These characteristics were seen in glands from both sexes and in both control animals and those receiving diethylstilbestrol. So contradictory were these appearances that we could not look upon them as being associated with diethylstilbestrol treatment or the lack of it, nor did they seem to be associated with castration.

Much more satisfactory results attended our study of the vacuoles in the zona fasciculata. These sharply defined cavities are readily distinguished from more diffuse patches of lighter protoplasm in this part of the cortex. In the males the vacuoles are sparse and located almost wholly in the peripheral part of this region, in a band below the zona glomerulosa; only occasionally are they found in the deeper zones. In the females however they are far more numerous and widespread, extending through the entire zona fasciculata to the zona reticularis. While these sex differences are quite evident, there is a large amount of variation in the number and distribution of these vacuoles.

Treatment with diethylstilbestrol brought about a marked increase in these vacuoles in both sexes until this portion of the cortex was found to be filled with them in some of the females. On the other hand, while this hormone brought about a decided increase in vacuolation in the males, it did not cause it to reach the degree found in treated females. With all the increase of vacuolation produced in the males by diethylstilbestrol there is still a tendency for it to be predominantly peripheral, as in the control males.

A very considerable number of investigators have found that castration of the rat, at least in the female, causes a diminution in the weight of the adrenal cortex, generally attributed to the zona reticularis (1, 7, 8, 10, 11, 12, 14, 23, 31). Pinto (14) finds that castration causes an increase in the weight of the adrenal glands of the male. Upon this point we have no right to draw conclusions from our small number of castrated animals, since this phase of our study was purely exploratory. The data in the table show that our work offers no support to this view.

DISCUSSION OF RESULTS AND CONCLUSIONS

In the review of literature the usual diversity of results is evident, and we shall not make any critical comments or attempt to reconcile them. Difference of opinion is found in every field of research. Our results are in full accord with those of the many investigators who have found that treatment with estrogens causes enlargement of the adrenal cortex. The work shows that this effect is produced to an approximately equal degree in males and females. In this connection there may be some significance in the fact that diethylstilbestrol treatment to the degree employed in these experiments causes a reduction in the testes to one-fourth of their normal weight and inhibits the development of the corpora lutea of the ovaries, in this way at least in part eliminating the influence of the gonads. In these changes we naturally expect the pituitary gland to play a rôle. Therefore, it is interesting to consider the work of Pinto (14). In his experiments upon rats he demonstrated the well-known fact that hypophysectomy causes a great reduction in the weight of the adrenal

glands. This could be partially compensated by injection of the anterior lobes of 5 pituitary glands per day. When injections of a large amount of estradiol benzoate were made into hypophysectomized rats there was no effect upon the adrenal gland unless anterior pituitary material was injected along with it; thus it is clear that the injection of estrogens into the hypophysectomized animals has, in itself, little if any effect upon the adrenal glands. Pinto (14) found, however, as so many others have observed, that administration of estrogens to animals with normal pituitary glands does cause marked increase in the weight of the adrenal glands. It is well known that poisons and other unfavorable influences bring about marked enlargement of the adrenal gland, and it might be argued that treatment with diethylstilbestrol is merely another such non-specific influence. Against this objection we might urge the fact that other estrogens produce the same effect, while the chemically similar androgens do not do so. This argument is not wholly convincing, although it is suggestive, but we must leave the objection unanswered for the present at least.

Our work is of significance in that we have used the guinea pig while practically all of the work in this field has been done upon the rat. It furthermore adds to the evidence that has accumulated in this field in which there has been much diversity of opinion.

SUMMARY

1. We have shown that oral administration of diethylstilbestrol to guinea pigs of both sexes causes definite enlargement of the adrenal cortex.

2. Female guinea pigs have a greater number of vacuoles extending deeper into the zona fasciculata than do the males. In both sexes, treatment with diethylstilbestrol causes a great increase in vacuolation, but their arrangement in the males is still predominantly peripheral.

3. Castration experiments upon a small number of animals failed to show any change in the weight of the adrenal glands.

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ACTION OF SOME SYNTHETIC STEROIDS IN ADRENALECTOMIZED IMMATURE RAT

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THE CAPACITY of progesterone to maintain growth and life in the immature adrenalectomized rat has been well established (1–4). Furthermore, Clausen (5) has reported that progesterone causes atrophy of the adrenal in the intact rat, an effect that has been observed with certain adrenal steroids, namely Kendall's compounds *A* and *B* and cortin (6). However, it does not protect the rat against water intoxication (1) nor is it active in the muscle test of Ingle (7). It is noteworthy that certain adrenal steroids which are effective in maintaining life in adrenalectomized animals do not promote muscular efficiency.

Gaunt (8) attempted to maintain adrenalectomized immature rats with pregneninolone in dosages of 1.7 mg. by injection in propylene glycol and 3.0 mg. daily, mixed with the food. This treatment not only failed to maintain life, but also elicited toxic reactions. He was also unable to prevent signs of adrenal insufficiency in a ferret given 10 mg. of pregneninolone by mouth each day.

Waterman et al. (9) reported that Δ^5-pregnene-3-ol-20-one-21-ol-acetate was inactive in the swimming test with rats. Selye (10), on the other hand, reported that 2 mg. per day of Δ^5-pregnene-3-ol-20-one-21-ol-acetate maintained life in the adrenalectomized immature rat and prevented hemoconcentration, decrease in blood chlorides, hypoglycemia and a rise in nonprotein nitrogen. Although no data were given he stated that growth occurred in adrenalectomized rats which were treated with the substance. He also reported that Δ^5-pregnene-3-ol-20-one showed some capacity to maintain life in such animals.

These two compounds are of especial interest since they can be oxidized directly into desoxycorticosterone acetate and progesterone, respectively, *in vitro*. Indeed, they are the immediate precursors of the latter compounds in widely used chemical syntheses (11). Pregneninolone (anhydrohydroxy-progesterone, ethinyl testosterone) and Δ^5-pregnene-3-ol-20-one-21-ol acetate have also been found to be inactive in the Ingle (7, 12) muscle-test.

MATERIAL AND METHODS

The rats used in this study were immature males of the Sprague-Dawley strain purchased from a local dealer. Bilateral adrenalectomy was performed in one stage under ether anesthesia through a single dorsal skin incision. All animals were maintained on 'Nurishmix'[2] pellets and tap water, given *ad libitum*, for several days before and throughout the experiment.

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[2] Pratt Food Company, Hammond, Ind.

The daily dosage of each steroid,[3] with the exception of pregneninolone, was dissolved in 0.2 cc. of sesame oil and injected in two equal portions. Treatment of surviving animals was continued for 14 days. Since pregneninolone is soluble in few liquids and is unstable in solution,[4] it was administered by stomach tube once daily as a freshly prepared suspension in 2 cc. of 20 per cent gum acacia. The suspension was prepared by crushing the requisite number of 10-mg. tablets in a mortar and

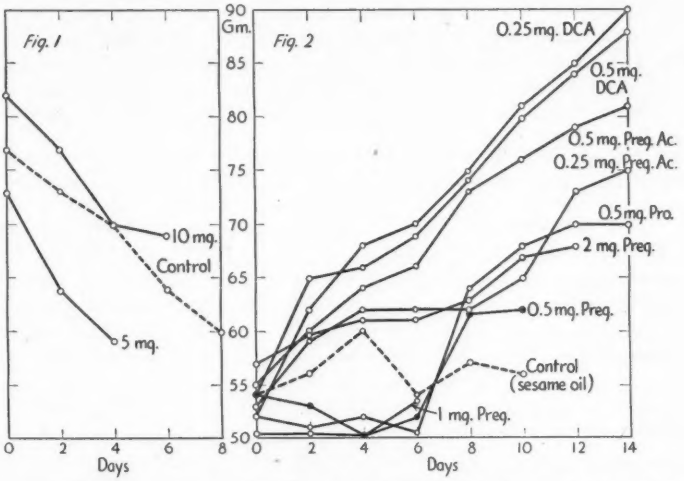

Fig. 1. GROWTH OF ADRENALECTOMIZED MALE RATS given pregneninolone orally. Control, 2 cc. of 20% gum acacia daily; 5 mg. indicates 5 mg. of pregneninolone in 2 cc. 20% gum acacia daily; 10 mg. indicates 10 mg. of pregneninolone in 2 cc. 20% gum acacia daily.

Fig. 2. GROWTH OF ADRENALECTOMIZED MALE RATS given subcutaneous injections of various steroids in oil. *Control*, 0.2 cc. sesame oil daily; *0.25 mg. DCA*, 0.25 mg. of desoxycorticosterone acetate daily; *0.5 mg. DCA*, 0.5 mg. of desoxycorticosterone acetate daily; *0.25 mg. Preg. Ac.*, 0.25 mg. of Δ^5-pregnene-3-ol-20-one-21-ol-acetate daily; *0.5 mg. Preg. Ac.*, 0.5 mg. of Δ^5-pregnene-3-ol-20-one-21-ol-acetate daily; *0.5 mg. Pro.*, 0.5 mg. of progesterone daily; *0.5 mg. Preg.*, 0.5 mg. Δ^5-pregnene-3-ol-20-one daily; *1 mg. Preg.*, 1 mg. Δ^5-pregnene-3-ol-20-one daily; *2 mg. Preg.*, 2 mg. Δ^5-pregnene-3-ol-20-one daily.

slowly adding the solution of gum acacia. Controls were given 2 cc. of 20 per cent gum acacia by stomach tube each day.

Animals which failed to succumb to adrenal insufficiency following the withdrawal of treatment are not included in the data which are presented.

RESULTS

The oral administration of either 5 or 10 mg. daily of pregneninolone failed to lengthen survival time or to improve the growth rate of immature adrenalectomized rats. Δ^5-pregnene-3-ol-20-one-21-ol-acetate in 0.5 mg. daily induced growth which almost equalled that obtained with 0.5 mg. of desoxycorticosterone acetate (fig. 2, table 1). The survival rates were also comparable since 11 of 12 rats which received the former substance and all 7 animals which were treated with desoxycorticosterone acetate survived the 14-day experimental period. When the dosage of these

[3] We wish to thank Dr. Ernst Oppenheimer, Ciba Pharmaceutical Products, Inc., Summit, N. J., for generous supplies of desoxycorticosterone acetate, progesterone, pregneninolone, Δ^5-pregnene-3-ol-20-one and Δ^5-pregnene-3-ol-20-one-21-ol-acetate.

[4] Personal communication from Dr. Ernst Oppenheimer.

two compounds was halved a marked difference between their effects appeared. In animals which received 0.25 mg. of desoxycorticosterone acetate, growth and survival was comparable to that found with 0.5 mg. of the same substance. On the other hand, only 2 of 11 rats receiving 0.25 mg. of Δ^5-pregnene-3-ol-20-one-21-ol-acetate survived 14 days. The re-alignment of the curves for animals on this substance, after the eighth day is due to the good growth of the 2 surviving animals.

The injection of 0.5, 1 or 2 mg. daily of Δ^5-pregnene-3-ol-20-one failed to lengthen survival time or to increase growth (fig. 2, table 1).

The comparative activity of progesterone under our experimental condition was studied in a group of 7 animals. In this series the administration of 0.5 mg. daily resulted in the survival of only one animal for 14 days. This single animal showed very poor growth. This is not surprising since it has been shown in other experiments that

TABLE 1. SURVIVAL OF IMMATURE ADRENALECTOMIZED MALE RATS TREATED WITH SYNTHETIC STEROIDS

Treatment	Daily Amount	Number of Animals	Number Surviving 14 Days	Average Survival, days[1]
Control, 20% acacia[2]	2 cc.	5	0	5
Pregnenin-17-ol-3-one[2]	5 mg.	5	0	4
	10 mg.	6	0	5
Control, sesame oil	0.2 cc.	15	0	5
Δ^5-pregnene-3-ol-20-one	0.5 mg.	5	0	6
	1 mg.	7	0	4
	2 mg.	6	0	7
Progesterone	0.5 mg.	7	1	8
Δ^5-pregnene-3-ol-20-one-21-ol-acetate	0.5 mg.	12	11	12
	0.25 mg.	11	2	10
11-desoxycorticosterone-21-acetate	0.5 mg.	7	7	
	0.25 mg.	9	9	

[1] Animals which survived throughout the 14-day experimental period are omitted from the figures in this column.

[2] *Per os*, all others, subcutaneously.

a minimum of 1 mg. daily is required to maintain life and to promote satisfactory growth.

DISCUSSION

Pregneninolone has many interesting properties in addition to its high oral potency in the production of a progestational endometrium. It is doubtful if its effect on the estrogen-primed endometrium in the human female (13) is due to conversion to progesterone since Hamblen et al. (14) have shown that there is no rise in the urinary excretion of the pregnanediol complex following the oral administration of pregneninolone. The injection of progesterone under the same conditions produces a rise in the urinary titre of the pregnanediol complex. Pregneninolone, like progesterone, shows a very mild androgenic action in the capon, castrated rat and in the young chick. On the other hand, while progesterone inhibits or prevents the cornification of the vagina produced by estrogens in the castrate, pregneninolone, orally or injected, cornifies the vagina of the castrated rat or mouse, and is slightly estrogenic when given to women in very large amount. That pregneninolone, unlike progesterone, does not inhibit uterine motility has been shown by the studies *in vitro* of Burge (15). Courrier and Jost (16) reported that very large doses of pregneninolone maintained pregnancy in the castrated rabbit, while Krohn and Harris (17) and Frank et al. (18) report that oral pregneninolone was beneficial in patients with habitual and

threatened abortion. Cohen and Stein (19) found that trauma produces deciduomata and that deciduomata so produced are atypical.

In view of the interesting properties of pregneninolone, including certain similarities to progesterone, we were prompted to investigate its capacity to maintain life in the adrenalectomized immature rat. Since the oral progestational activity of preneninolone is about one-fifth to one-tenth (20, 21) that of progesterone, 5 and 10 mg. of pregneninolone were selected as the doses for oral tests. These levels were ineffective in maintaining growth or prolonging life.

Selye (22) has discussed Δ^5-pregnene-3-ol-20-one at some length and states that only it and Δ^5-androstene-3(B), 17(a)-diol share the cornifying action which characterizes the estrane derivatives. Despite this action Δ^5-pregnene-3-ol-20-one possesses the interesting property of having very weak androgenic activity, yet it is readily able to inhibit the testicular atrophy otherwise produced by estradiol. In the same discussion Selye lists Δ^5-pregnene-3-ol-20-one-21-ol-acetate as possessing the same degree of 'folliculoid' activity as Δ^5-pregnene-3-ol-20-one, but regards it as lacking in androgenic activity.

Dr. W. J. Eversole of the Department of Biology, Washington Square College, New York University, was kind enough to test Δ^5-pregnene-3-ol-20-one-21-ol-acetate for its activity in the 'water intoxication' test (23). His results indicated that the compound is of about the same potency in this test as desoxycorticosterone acetate. The 'median 11-hour water excretion' for 6 animals given 3 mg. of Δ^5-pregnene-3-ol-20-one-21-ol-acetate in sesame oil was 90.1 per cent. It is noteworthy that the administration of 7.5 mg. of progesterone during the period of the test failed to protect adrenalectomized rats from an excess of water (1).

Our data show that pregneninolone and Δ^5-pregnene-3-ol-20-one are unable to maintain life or to promote growth in the adrenalectomized immature rat at dosages of 5 and 10 mg. and 0.5 to 2 mg. daily, respectively. On the other hand, Δ^5-pregnene-3-ol-20-one-21-ol-acetate shows a degree of activity which lies somewhere between that of desoxycorticosterone acetate and progesterone. Our results on the study of the activity of this compound for life maintenance are in general agreement with those of Selye (10), although they indicate a somewhat higher order of potency. Furthermore, it was demonstrated that it will produce satisfactory growth in adrenalectomized immature rats.

SUMMARY

Pregnenin-17-ol-3-one in daily dosages of 5 or 10 mg. *per os* failed to increase the survival time of adrenalectomized, immature, male rats.

Δ^5-Pregnene-3-ol-20-one in amounts up to 2 mg. per day parenterally in sesame oil failed to prolong life in adrenalectomized, immature, male rats.

Δ^5-Pregnene-3-ol-20-one-21-ol-acetate is markedly potent and falls between progesterone and desoxycorticosterone acetate in its ability to produce growth and to lengthen the survival time of adrenalectomized, immature, male rats.

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CHEMICAL STUDY OF TISSUE CHANGES IN ADRENAL INSUFFICIENCY AND TRAUMATIC SHOCK[1]

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THE INCREASED incidence in wartime of secondary, traumatic or wound shock demands that every effort be directed to the solution of what still is a mysterious malady. Injury to non-vital parts, with little hemorrhage, may lead to death in a few hours. Similarities in certain signs and symptoms of adrenal insufficiency and of shock have been pointed out by a number of observers, and most emphatically in the last decade by Swingle and his co-workers (1).

The chief physiological similarities are *a*) diminished plasma volume and reduced blood pressure, *b*) failure of the vascular system to mobilize fluid or to retain injected saline and, as pointed out more recently, *c*) an increase in serum potassium (2, 3). The blood-sugar level and heart rate may differ in the two conditions: in shock, both are usually elevated; in adrenal insufficiency, the blood-sugar level is lowered and, in our experience, the heart rate is decreased (4, 5). The pathological similarities have been described in detail by Moon (6). The essential feature, common to both conditions, is congestion of the abdominal viscera. This is especially marked in the mucosa of the gastrointestinal tract but other tissues, *e.g.*, pancreas, kidneys and liver, are also nearly always very congested, particularly in adrenal insufficiency.

It was felt that tissue analyses might disclose further similarities or differences which would help identify or differentiate the two conditions. Closer identification of shock with adrenal insufficiency might suggest a logical basis for the use of cortical hormones in the treatment of shock. Certainly, at present, theoretical grounds are inadequate for the application of such therapy. Too, the use of cortical hormones in shock has not given impressive results, except in adrenalectomized animals.

While there have been a number of studies on tissue changes in animals suffering from adrenal insufficiency, reports of similar studies on animals in shock have not been seen. In adrenal insufficiency, significant changes in the concentration of potassium in tissues have been revealed (7–10). Phosphorus, although predominantly an intracellular ion also, showed less marked variations. In human tissues, Cullen, Wilkins and Harrison (11) found a stability in the ratio of phosphorus to potassium in a given tissue. In an effort to throw further light on changes in these ions in various tissues in both adrenal insufficiency and traumatic shock, the present study was undertaken.

METHODS AND RESULTS

General. Tissues of rats and dogs in normal health, in adrenal insufficiency and in shock have been studied. Samples of muscle, liver and small intestine were obtained under nembutal anesthesia. In dogs, pancreas and heart muscle were also obtained.

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[1] This study was part of an investigation carried on under a grant from the National Research Council of Canada.

All tissue samples were analyzed for water, blood, potassium and phosphorus content. Estimations of blood-serum sodium and potassium and determinations of packed cell volume were made in some cases.

Adrenalectomy was performed by standard methods which we have used over a period of years. The rats were shocked by the technique of Noble and Collip (12) after brief preliminary etherization; the dogs, by 1000 to 1400 light blows on each thigh with a rubber mallet, while under pentothal anesthesia, according to the method employed by Best and Solandt (13) and by Kendrick, Essex and Helmholz (14).

Tissues were secured from adrenalectomized animals when marked signs of adrenal insufficiency were present: in rats, 5 to 7 days after adrenalectomy; in dogs, 6 to 8 days after withdrawal of cortical hormone used in effecting maintenance. Shocked rats and dogs were on the verge of death when tissue samples were taken, 2 to 4 hours and 1.5 to 6 hours, respectively, after traumatization. For blood-serum potassium studies other rats and dogs were used.

Chemical. Tissues were removed quickly, weighed and dried, then treated by the method of Cullen and Wilkins (15). Aliquots of a nitric acid digest were used for analyses, and the tissues of each animal were analyzed individually. Potassium in the tissues was estimated by the technique described by Shohl and Bennet (16); phosphorus by the technique of Fiske and Subarrow (17); blood content of tissues by the technique of Manery, Danielson and Hastings (18). Blood sodium was determined by the method of Butler and Tuthill (19) and blood potassium by the method of Shohl and Bennett (16). Packed cell volume determinations were made on heparinized blood. Water analyses were made in order to make sure that any change found in the concentration of electrolytes was due to a change in these elements per se. The blood content of various tissues was determined for the same reason but the results were not found to be significantly affected.

Tissue Analyses in Rats

The tissues of rats in adrenal insufficiency and shock showed significant deviations from the normal in the water, potassium and phosphorus content. The direction of the major changes in electrolytes was different in the two conditions. Water changes of sufficient magnitude to obscure a small increase in potassium occurred in muscle only of shocked rats. The data are summarized in table 1 and illustrated in figure 1. The chief changes observed were as follows.

Muscle showed slight increases in the concentration of both potassium and phosphorus in adrenal insufficiency. In shock, the concentration of both these ions was less than normal but the increase of water was greater than the decrease in potassium so that calculation on the basis of the water content of normal muscle revealed a net gain of potassium of about 1 per cent. By similar calculation, phosphorus showed only a negligible decrease.

Liver showed a marked potassium increase and slight phosphorus decrease in adrenalectomized rats. Large decreases in content of both of these ions were found in shocked rats.

Small intestine, on analysis, showed a slight increase of potassium and phosphorus in adrenal insufficiency. While not in themselves so striking, these changes formed a marked contrast to the decrease in both potassium and phosphorus found in the gut of shocked rats. This decrease was similar to that seen in the liver.

Blood potassium estimations were not made in this series of adrenalectomized rats since previous experiments (8) have shown a potassium increase in the blood

TABLE 1. WATER, POTASSIUM AND PHOSPHORUS OF MUSCLE, LIVER AND SMALL INTESTINE
(The results are expressed per kilogram of fresh tissue.)

	Normal Controls	Adrenal Insufficiency	Shock
Number of Animals (rats)	24	21	18
Muscle[1]			
Water, gm.	753	766	769
Potassium, millimols	96.5	97.5	95.5
Phosphorus, millimols	78.9	79.4	77.1
Liver			
Water, gm.	735	748	747
Potassium, millimols	84.2	87.9	75.1
Phosphorus, millimols	119.0	114.2	108.0
Small intestine[2]			
Water, gm.	799	796	802
Potassium, millimols	87.1	87.8	80.6
Phosphorus, millimols	104.2	106.9	101.1

[1] Abdominal muscle.
[2] Whole of the small intestine.
Note: In this table, and in subsequent tables, values for potassium and phosphorus have been expressed as millimols per kilogram of fresh tissue. These are convertible to milligrams per kilogram by multiplying by the following factors: for potassium, by 39.1; for phosphorus, by 31.

of rats in adrenal insufficiency, values of 29 to 32 mg. per cent having been found. The results of serum potassium analyses in 20 shocked rats are summarized in table 2 in which other data pertinent to the degree of shock in the different groups are also noted. These observations show that the degree of increase in the serum potassium in shocked rats coincided closely with *a*), the clinical degree of shock existing at the

TABLE 2. SERUM POTASSIUM LEVELS AND CORRELATED FINDINGS IN SHOCKED RATS[1]

Number of Rats[2]	Clinical Degree of Shock[3]	Serum Potassium	Amount of Blood Easily Obtained[4]	Hemolysis	Degree of Congestion of Viscera
		mg. %	cc.		
2	None (controls)	17	7.0	None	Normal
1	Very mild	15.8	5.0	Very slight	Caecum: very slight
1	Very mild	18.6	4.5	Very slight	Caecum: slight
2	Mild	19.5	3.0	Slight	Caecum: moderate in one; severe in other
2	Mild	24.4	Not noted	Moderate	Caecum: moderate in both; small intestine: slight in one; moderate in other moderate in both
2	Moderate	20.4	2.4	Moderate	Caecum: severe in both; small intestine:
5	Severe	29.5	About 1	Severe	Caecum, large and small intestine: moderate
5	Severe	37.1	About 1	Severe	Caecum, large and small intestine: severe; pancreas: moderate; fallopian tubes: moderate

[1] Weight of rats 140 to 172 grams.
[2] When more than one rat was used, serums were pooled for analysis.
[3] *Very mild:* short period of inactivity after trauma; animals quite frisky when killed; *mild:* period of inactivity after trauma not succeeded by friskiness; *moderate:* period of inactivity merged imperceptibly into semi-comatose state; *severe:* inactivity proceeded to prostration.
[4] Blood obtained from aorta. Sample drawn 3 to 6 hours after trauma in mild and moderately shocked rats, and 10 minutes to 3 hours after trauma in severely shocked animals.

time the blood was drawn; *b*), the amount of blood that could be obtained easily from the aorta in an oiled syringe; *c*), the degree of hemolysis of the serum obtained; *d*), the degree of congestion of the viscera observed directly after the blood sampling.

Since hemolysis was present despite the exercise of extreme care in drawing the blood, it was concluded that it existed *in vivo*. This conclusion was supported by the observation that some of the rats which survived a few hours after being shocked

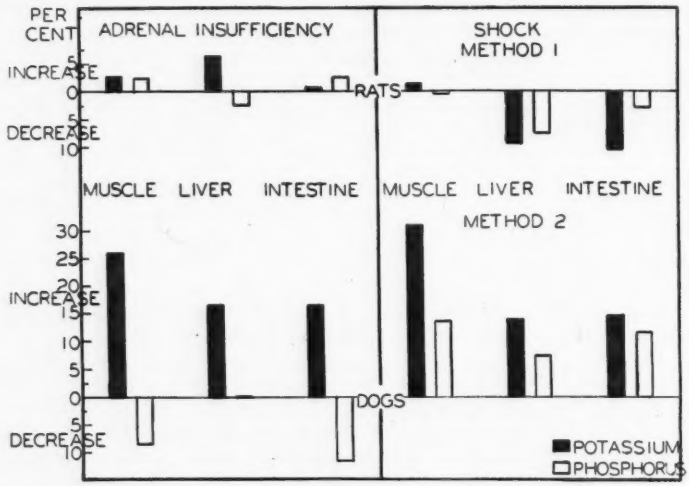

FIG. 1. CHANGES IN POTASSIUM AND PHOSPHORUS IN TISSUES of rats and dogs in adrenal insufficiency and shock, illustrated as percentage deviation from average of normal controls. (Corrected for changes in water.)

voided pink urine, the color presumably being due to hemoglobin. As the red blood cells of the rat have a concentration of potassium 20 times that found in serum (20), it would appear that the hemolysis contributed, in some degree, to the increase of potassium in serum.

It was also observed that the blood of shocked rats was markedly concentrated, as measured by the proportion of serum to cells in the centrifugate and by the hemoglobin percentage. The average hemoglobin percentage in 17 shocked rats was found to be 35 per cent higher than that of 9 normal rats.

Tissue Analyses in Dogs

The tissues of adrenalectomized and shocked dogs exhibited an even more marked departure from the average figures obtained on normal controls than was found in rats. In adrenal insufficiency the potassium changes were in the same direction as in rats but, in the shocked dogs, large increases of potassium and phosphorus were found in contrast to decreases in these elements in shocked rats. The data are listed in tables 3 and 4 and illustrated in figures 1 and 2. In no case were the water changes sufficiently great to affect the direction of the changes in the tissue electrolytes studied. The following observations were made.

Muscle of dogs showed a marked increase in the concentration of potassium in

both adrenal insufficiency and shock. Phosphorus was decreased in adrenal insufficiency but increased in shock.

Liver exhibited a marked increase in potassium in both experimental conditions. In adrenal insufficiency there was a negligible decrease in phosphorus; in shock phosphorus was increased.

Small intestine showed increases in potassium in both adrenal insufficiency and shock. In adrenal insufficiency, phosphorus was decreased but increased in shock.

TABLE 3. WATER, POTASSIUM AND PHOSPHORUS CONTENT OF MUSCLE, LIVER, SMALL INTESTINE, PANCREAS AND HEART (LEFT VENTRICLE)
(The results are expressed per kilogram of fresh tissue)

	Normal Controls	Adrenal Insufficiency	Shock
Number of animals (dogs)	6	4	4
	Muscle[1]		
	Average	*Average*	*Average*
Water, gm.	732.0	768.0	738.0
Potassium, millimols	71.8	86.2	93.2
Phosphorus, millimols	66.6	58.2	75.1
	Liver		
Water, gm.	708.0	733.0	713.0
Potassium, millimols	66.1	74.5	74.7[2]
Phosphorus, millimols	98.4	95.1	105.0
	Small intestine, duodenum		
Water, gm.	781.0	773.0	754.0
Potassium, millimols	77.5	91.3	92.1[2]
Phosphorus, millimols	82.2	73.9	95.2
	Pancreas		
Water, gm.	707.0	740.0	662.0
Potassium, millimols	64.2	90.0	79.3[2]
Phosphorus, millimols	125.3	137.5	137.7
	Heart, left ventricle		
Water, gm.	773.0[3]	782.0	772.0
Potassium, millimols	75.6[3]	83.7	97.4
Phosphorus, millimols		74.8	84.8

[1] Foreleg muscle.
[2] Average of three analyses only.
[3] Data calculated from report of Muntwyler et al[10].

Pancreas showed marked increases in the concentration of potassium and phosphorus in both conditions; the potassium increase was exceptionally great in adrenal insufficiency.

Heart muscle analyses were not obtained in normal dogs, since the decision to include this tissue was made only after the other control data were obtained. Potassium and phorphorus concentrations were considerably higher in the heart of shocked dogs than in adrenal insufficiency. By comparison with data calculated from Muntwyler and his collaborators (10), the potassium in the heart of adrenalectomized dogs was significantly elevated.

Figure 1 gives a graphic comparison of the changes in potassium and phosphorus found in muscle, liver and intestine of dogs in adrenal insufficiency and shock. The results have been corrected for changes in the water content of the

TABLE 4. WATER, POTASSIUM AND PHOSPHORUS CONTENT OF MUSCLE, LIVER, SMALL INTESTINE, PANCREAS AND HEART (LEFT VENTRICLE)

(The results are expressed per kilogram of fresh tissue)

	Normal Controls		Adrenal Insufficiency		Shock	
Number of animals (dogs	6		4		4	
	Muscle[1]					
	Maximum	*Minimum*	*Maximum*	*Minimum*	*Maximum*	*Minimum*
Water, gm.	763.0	697.0	774.0	764.0	774.0	702.0
Potassium, millimols	83.9	63.1	89.8	81.5	100.1	89.8
Phosphorus, millimols	71.9	60.3	59.5	57.3	76.8	70.7
	Liver					
Water, gm.	736.0	596.0	744.0	715.0	706.0	718.0
Potassium, millimols	71.2	57.5	78.1	66.4	80.0	70.1
Phosphorus, millimols	107.4	93.8	96.4	93.5	117.9	94.6
	Small intestine, duodenum					
Water, gm.	791.0	776.0	788.0	742.0	774.0	726.0
Potassium, millimols	81.5	69.0	97.4	88.9	98.5	74.5
Phosphorus, millimols	88.2	76.1	80.3	69.6	99.5	85.4
	Pancreas					
Water, gm.	786.0	648.0	749.0	732.0	706.0	630.0
Potassium, millimols	67.3	60.4	99.7	83.8	91.7	68.5
Phosphorus, millimols	135.0	120.5	154.5	127.1	139.5	126.5
	Heart, left ventricle					
Water, gm.			788.0	773.0	780.0	767.0
Potassium, millimols			89.9	76.2	97.7	96.8
Phosphorus, millimols			66.1	58.2	97.6	72.1

[1] Foreleg muscle.

tissues and are expressed as percentage deviation from the normal controls. In figure 2 the changes found in pancreas and heart are similarly illustrated. Data from the report of Muntwyler (10) have been used as a basis of comparison. No change has been noted in the phosphorus concentration in the heart of dogs in adrenal insufficiency since no satisfactory data on normal dogs was available for comparison.

Blood-serum sodium and potassium analyses in dogs in adrenal insufficiency and shock are summarized in table 5. The decrease in serum sodium and increase in potassium found in adrenal insufficiency are characteristic of this condition. There was no significant change found in the serum sodium in shocked dogs. The first figure listed in table 5 for serum potassium in shocked dogs (21.4 mg. per cent) represents the average of values obtained when signs of shock were definitely established. The range of values found was 16.2 to 24.5 mg. per cent. At time of death,

TABLE 5. TYPICAL BLOOD VALUES IN EXPERIMENTAL ADRENAL INSUFFICIENCY AND SHOCK IN DOGS

Number of Dogs	Condition	Serum	
		Sodium	Potassium
		mg. %	mg. %
5	Normal	330	17.3
4	Adrenal insufficiency	298	27.3
4	Shock	327	21.4 (at death 31.6)

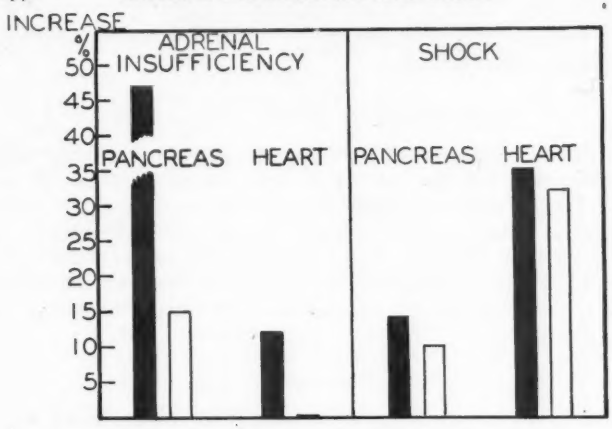

FIG. 2. CHANGES IN POTASSIUM AND PHOSPHORUS IN PANCREAS AND HEART of dogs in adrenal insufficiency and shock, illustrated as percentage deviation from average of normal controls in pancreas and calculated from data of Muntwyler as percentage deviation from control figure in heart muscle. (Corrected for changes in water.)

serum potassium averaged 31.6 mg. per cent, values ranging from 29 to 38 mg. per cent. The progressive rise in the concentration of potassium in the serum of shocked dogs is illustrated in figure 3. The terminal large increase is characteristic. Changes in the volume of packed red blood cells occurred inconstantly in shock. When changes did occur they were of less marked degree than in adrenal insufficiency in which a 10 to 30 per cent increase in the volume of packed red blood cells was found. In shock, decreases in packed cell volume were observed at times. No relation between the serum-potassium level and degree of congestion of the viscera was observed in shocked dogs. Post-trauma blood samples were always hemolyzed. There did not appear to be any gradation in the severity of the hemolysis as in the shocked rats. Contrary to the condition in rats, the concentration of potassium in the red blood

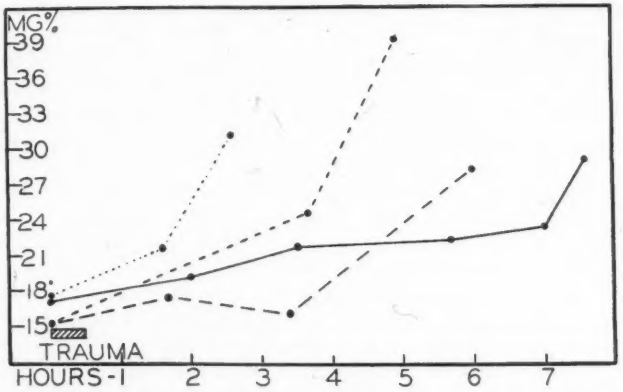

FIG. 3. INCREASE IN SERUM POTASSIUM IN SHOCKED DOGS. Terminal value in each case determined from heart blood at time of death.

cells of the dog is approximately the same as in serum; hence, hemolysis in the dog does not increase serum potassium.

Pathological Changes in Shocked Rats and Dogs

The congestion of abdominal viscera was constantly severe in rats dying of shock; it was more variable and less intensive in dogs. A little blood was often observed about the nostrils of rats and, occasionally, a hematoma about an eye, but no other gross evidence of blood loss or tissue trauma was seen. Considerable swelling of the traumatized limbs of dogs was constantly found. At autopsy, the subcutaneous tissues and muscle in the traumatized region were edematous and blood-stained. Ten to 25 cc. of free sanguinous material and small blood clots were found in the crushed muscle mass.

DISCUSSION

The cause of the potassium increase in tissues and in blood serum in adrenal insufficiency in both rats and dogs is renal retention (9). It is not clear why the phosphorus changes were different in the two species.

The reason for the decreased concentration of potassium and phosphorus in liver and intestine of shocked rats is not obvious. If it were a direct effect of trauma, it might be expected that muscle would show a similar loss. This was not found. It seems probable that these chemical changes in liver and intestine may be important factors contributing to the state of shock. Noble and Collip (12) found that eviscerated rats withstood traumatization by their method better than intact animals. Certain experiments reported in the literature suggest how the viscera play an important part in the production of shock caused by trauma. It has been shown by Freeman and his associates (21, 22), *a*) that adrenalin infusions and *b*), that excitation of the sympathetic nervous system will produce shock in cats. These influences, whatever else they may do, operate to release potassium from the liver (see Scudder, 2, for references) and possibly from the intestine also. Certainly, in our shocked rats, the experimental conditions were appropriate for the production of excitation of the sympathetic nervous system and attendant adrenalin secretion. On the basis of the experimental work referred to, this might be expected to lead to shock and loss of potassium from the liver.

Whether the potassium lost from the liver and intestine of the shocked rats in this study was a cause or an accompaniment of the shock cannot be said with certainty. It was, however, sufficient to account for the slight rise in potassium in muscle and for a rise of approximately 8 mg. per cent of potassium in extracellular fluids, including serum. This does not account wholly for the degree of elevation of serum potassium found in our severely shocked rats. The extent of the hemolysis of the red blood cells was probably sufficient to make up the difference.

In the shocked dogs, the increase in both potassium and phosphorus found in all five tissues examined suggests that both of these elements were liberated from the traumatized muscle and spread throughout the body. It is difficult to account for the large increases in potassium and phosphorus on the basis of the amount of muscle traumatized. Analyses performed on muscle samples obtained by biopsy from the same animal before and after traumatization might show smaller differences than those reported here. Nevertheless they were so gross that there can be little doubt as to the direction of the change; the magnitude is of less significance. The source of the elevated serum potassium is probably the same as for the tissues, although the relatively higher potassium values in serum at the time of death than in samples

taken earlier suggest, in confirmation of Manery and Solandt (4), that the final high value may have been a terminal phenomenon, e.g., liberation from liver.

Since Scudder (2) has laid so much emphasis on the elevation of blood potassium in shock, it is pertinent to point out that the potassium values found at death are not sufficient *per se* to cause death; higher values have been encountered in experimental anuria (23) and in potassium injection experiments (24) before death occurred. Moreover, high terminal potassium values may be a result of death processes. In assessing the importance of elevated serum potassium in shock, the rate of increase must be considered; this probably is more important than the actual level attained. Recent investigations of Schamp (25) show that the amount of injected potassium, which Winkler and his associates (24) reported to be lethal for normal dogs, can be greatly exceeded if the infusion is made slowly. Furthermore, such dogs will withstand for several days an elevation of serum potassium to a level equal to, or greater than, that seen in adrenal insufficiency, and to a considerably greater level than that seen, except terminally, in shock. Obviously potassium is not the sole factor in shock. Possibly an increased level of potassium in the blood is more significant physiologically when there is a reduction in blood volume, as in shock and adrenal insufficiency. Then it may contribute to the failure of the animal to mobilize fluid from interstitial spaces into the blood stream by an effect on capillary function.

Two other points concerning potassium concentrations in tissues deserve mention. The first is that grossly different potassium concentrations in the tissues may be associated with markedly elevated serum potassium levels; and the second, that the congestion of the gastrointestinal tract is not always associated with an increased concentration of potassium in that tissue,—it is reduced in shocked rats.

The congestion of the abdominal viscera in shock must be due to some change common to both rats and dogs. The elevated serum potassium or prolonged vasoconstriction of vessels in the splanchnic region may be the cause. In adrenal insufficiency, there is evidence that the congestion may be due to a capillary defect resulting from the lack of adrenal cortical hormone (26, 27, 28). There is no evidence that this is a factor in shock.

Our investigation shows that two forms of trauma may lead to shock in laboratory animals. The common feature in shock produced by either method is elevated serum potassium. Fluid loss from blood at the site of trauma probably was an important factor in production of the shock in dogs. Hemoconcentration was not always present in these animals. In rats, there was no gross fluid loss into any of the tissues examined but hemoconcentration was present, indicating a generalized fluid shift from the blood stream. Since some of the rats that recovered passed loose stools, passage of fluid from the blood stream to gut may have occurred. The source of elevated serum potassium in dogs was apparently the peripheral traumatized areas and, in rats, the viscera and hemolyzed red blood cells. In dogs, direct trauma was the cause; in rats, trauma probably effected the changes in liver and intestine through the mediation of the sympathetic nervous system.

SUMMARY

Tissue analyses for potassium and phosphorus have been made on rats and dogs in adrenal insufficiency and shock, and on normal controls. The following significant changes were found.

a) Increases in potassium in muscle, liver and small intestine were observed in both rats and dogs in adrenal insufficiency; potassium increase was also found in the pancreas of dogs. The changes were considerably greater in dogs in all of the tissues examined.

b) In shocked rats, marked losses of potassium and phosphorus occurred in liver and small intestine.

c) In shocked dogs, marked increases in both potassium and phosphorus were found in muscle, liver, intestine, pancreas and heart.

d) In both shocked rats and dogs, blood analyses showed increases in serum potassium.

The increase in the concentration of potassium in tissues of animals in adrenal insufficiency is ascribed to renal retention. In shocked rats, the decrease of potassium in liver and intestine is attributed to an indirect effect of the trauma transmitted through the sympathetic nervous system. The increase of potassium in the blood serum of severely shocked rats may be accounted for by the visceral losses of this element and the occurrence of hemolysis. In shocked dogs, the increase in potassium and phosphorus found in the tissues and the increase of potassium in the blood serum are thought to be due to liberation of these elements from the traumatized muscle.

From this study it appears that the determination of the changes in concentration of potassium in tissues is chiefly important in indicating the source of increased potassium in the blood stream.

The importance of increased blood potassium in shock is discussed and it is concluded that, while it cannot be the sole factor in shock, in association with a reduced blood volume it may have a deleterious effect. The rôle of potassium in the production of visceral congestion in shock and adrenal insufficiency is considered.

To Professor Duncan Graham we are grateful for helpful criticism and encouragement. We are indebted to Dr. Jeanne Manery Fisher for the benefit of her advice and for assistance with some of the blood analyses. We also wish to acknowledge the kindness of Professors Best and Solandt in demonstrating a technique.

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EFFECT OF ADRENALECTOMY ON THE LACTOGENIC HORMONE AND INITIATION OF LACTATION[1,2]

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IT IS a well established fact that the adrenals are essential for normal lactation (1–4). Animals adrenalectomized during pregnancy may deliver normal litters, but they rarely lactate sufficiently to raise them (3, 5). Adrenalectomy is known to result in the upset of several important body functions, such as the maintenance of electrolyte and water balance and carbohydrate metabolism. These deficiencies alone might be considered sufficient to account for the failure of lactation following adrenalectomy. However, there is some evidence which indicates that adrenalectomy may also exert an adverse effect on the secretory activity of the anterior pituitary. It is this latter problem which will be considered here, and specifically, as it relates to the lactogenic function of the pituitary.

It is generally agreed that in Addison's disease, which is characterized by a dysfunction of the adrenal cortex, there is a degranulation and degeneration of the basophil cells of the anterior pituitary (6–8). Similar findings have been reported in the pituitaries of rats (9–11) and fowl (12) following adrenalectomy, and also in dogs maintained in a state of chronic adrenal insufficiency (13). Martin (9), as well as Shumacker and Firor (14) both agree that following adrenalectomy in rats there is a reduction in the gonadotropic content of the pituitary. There is also some evidence that the growth hormone of the pituitary is reduced in animals maintained for long periods in a state of chronic adrenal insufficiency (10). Grollman and Firor stated that some of the symptoms of chronic adrenal insufficiency in dogs could be remedied only by administering anterior pituitary extracts (13).

Inasmuch as the basophil cells of the pituitary are believed to be primarily affected by adrenalectomy, a less direct effect of adrenal absence might be expected on the lactogenic hormone, which is presumed to be secreted by eosinophils. Three groups of workers have presented evidence from which they have concluded that the lactogenic hormone is secreted by acidophilic cells of the pituitary (15–17). In cattle pituitaries, a greater concentration of lactogen was observed to be present in the peripheral zone in which there is a predominance of acidophilic cells (16, 17). It seemed desirable, therefore, to determine whether adrenalectomy would affect the production of the lactogenic hormone by the pituitary.

This laboratory has shown previously that *a*), estrogens can increase the lactogen content of the pituitary and *b*), that the lactogen content of the pituitary remains relatively low during pregnancy in most of the animals studied thus far, but increases by several hundred per cent following parturition. Would adrenalectomy prevent this increase in pituitary lactogen which can be secured experimentally with estrogen, and which occurs normally following parturition?

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EXPERIMENTAL PROCEDURE

One hundred and eleven albino and white-hooded female rats, and 29 male and female guinea pigs were used in these experiments. The suitability of our strain of albino rats for adrenalectomy studies was determined by adrenalectomizing a preliminary group of 19 rats (*group C*) and then observing the length of survival. The adrenals were removed through a lumbar incision in a one-stage operation. All of the animals died within 7 to 18 days, or on an average of 12.4 days after the operation, indicating that adrenal insufficiency in these rats developed within a reasonably short period of time. The rats averaged 188 gm. in body weight on the day of operation and 143 gm. on the day of death, losing an average of 24 per cent in body weight. When these animals died the pituitaries were removed, weighed, and kept frozen for assay. Another group (*D*) of control rats were adrenalectomized and killed 7 days after the operation, while a third adrenalectomized group (*E*) were injected subcutaneously with 200 I.U. of estrone daily for 5 days, beginning on the second day after the operation. This group of estrogen-treated rats were also killed on the 7th day after adrenalectomy. For comparative purposes, two groups of normal, intact albino rats were used, one group (*A*) serving as controls while those in the other (*B*) were injected subcutaneously with 200 I.U. of estrone daily over a 5-day period. The pituitaries of each group were preserved separately, and lactogen assays were made by the Reece-Turner pigeon method (18). One Reece-Turner unit of lactogen has been found to be equivalent to 0.045 international units of hormone (19).

Adrenalectomy in the guinea pig was performed in a two-stage operation. The right adrenal was removed first through an opening formed by removing the penultimate rib, and one to two weeks later the left adrenal was similarly removed. Since it was difficult to keep the guinea pigs alive after the operation without any treatment, they were given either one or two injections daily of 1.0 mg. of desoxycorticosterone acetate,[3] and also extra salt in the drinking water. Greater total dosages of desoxycorticosterone acetate than those used in this experiment have been shown to have no effect on the lactogen content of the pituitary in guinea pigs (20). Three groups of male and one group of female adrenalectomized guinea pigs were injected with 600 I.U. of estrone in corn oil daily for a maximum period of 5 days, and then killed. The pituitaries were removed for lactogen assay.

For the postpartum studies, both albino and white-hooded rats were adrenalectomized during the last week of pregnancy, and killed 48 hours after parturition. They were compared with intact female rats which were also killed 48 hours postpartum, as well as with a group of intact females which were killed in an advanced stage of pregnancy. In order to avoid the immediate effect of suckling in causing a discharge of lactogenic hormone from the pituitary (18), the litters were removed from the mother rats 15 hours before autopsy. The pituitaries of all of these rats were removed for lactogen assays and the mammary glands were grossly examined in the postpartum rats for an estimation of the degree of lactation. The stomachs of the pups were also examined for the presence of milk.

OBSERVATIONS

The effects of adrenalectomy on the lactogen content of the pituitaries of normal rats, and on the increased lactogen content of the pituitaries of estrogen-treated female albino rats are recorded in table 1. The 15 intact control rats (*A*) contained an average or 0.227 I.U. or 5.05 R.-T. U. of lactogen per pituitary, while the 19 adrenalec-

[3] The desoxycorticosterone acetate was supplied by Dr. Erwin Schwenk of the Schering Corp., Bloomfield, N.J.

tomized rats (C) had an average of 0.176 I.U. or 3.91 R.-T.U. per pituitary. On a body-weight basis, there was 27.5 per cent less lactogen in the pituitaries of the adrenalectomized than in the intact animals. The group of adrenalectomized rats (*D*) which were killed 7 days after the operation, assayed 0.169 I.U. or 3.75 R.-T.U. of lactogen per pituitary. This represents an average decrease in hormone content of 28.9 per cent, or about the same as in the first group of adrenalectomized rats.

TABLE 1. EFFECT OF ADRENALECTOMY ON NORMAL CONTENT AND ESTROGEN-INDUCED INCREASE OF LACTOGENIC HORMONE IN PITUITARIES OF FEMALE ALBINO RATS

Group, Treatment	Days Post-operative when Autopsied	No. of Rats	Ave. Body Wt. Before Treatment	Ave. Pituitary Weight	Ave. Lactogen Per Pit.	Ave. Reece-Turner Units			Ave. % Increase Lactogen per 100 gm. Body Wt.
						Per pit.	Per mg. of pit. tissue	Per 100 gm. body-weight	
			gm.	mg.	I.U.				
A, Normal controls	Unoperated	15	176	10.04	.227	5.05	0.50	2.87	
B, Normal + 1000 I.U. of estrone	Unoperated	10	173	12.26	.709	15.75	1.28	9.10	216.9
C, Adrenalectomized	12.4	19	188	9.30	.176	3.91	0.42	2.08	−27.5
D, Adrenalectomized	7.0	14	184	8.61	.169	3.75	0.40	2.04	−28.9
E, Adrenalectomized + 1000 I.U. estrone	7.0	14	185	11.93	.489	10.87	0.91	5.87	104.5

The average lactogen content in the pituitaries of the estrogen-treated intact rats (*B*) rose to 0.709 I.U. or 15.75 R.-T.U. which is 216.9 per cent more hormone than in the untreated intact rats (*A*), and this is also greater by some 50 per cent than the amount previously reported in the pituitaries of postpartum lactating rats (21, 22). In the adrenalectomized rats which received estrogen (*E*) the pituitary lactogen content rose to 0.489 I.U. or 10.87 R.-T.U. of lactogen, which represents an increase in hormone of 104.5 per cent.

The data on the adrenalectomized guinea pigs are given in table 2. Although most of the animals lived but a few days, the increase in the lactogen potency of the pituitary following estrogen treatment was considerable. The 4 male guinea pigs which lived to receive 2 injections of estrone (1200 I.U.), and the 2 male guinea pigs which got 3 injections of estrone (1800 I.U.), showed an average increase in pituitary lactogen of 195 and 174 per cent, respectively. The 2 male animals which survived 5 daily injections of estrone showed an increase in pituitary lactogen of 382 per cent. The female guinea pigs which were adrenalectomized and received estrogen showed an average increase of 59 per cent in pituitary lactogen as compared to the untreated adrenalectomized controls. Similar increases in pituitary lactogen have previously been reported in intact guinea pigs treated with estrogen over corresponding periods of time (23). However, inasmuch as most of the guinea pigs used in the present experiment survived but a few days, it was difficult to ascertain whether the full effects of adrenalectomy were operative on the pituitary.

TABLE 2. EFFECT OF ADRENALECTOMY ON THE ESTROGEN-INDUCED RISE IN LACTOGEN CONTENT OF PITUITARIES OF GUINEA PIGS

Group, Treatment	Sex and No. of Guinea Pigs	Days Post-operative When Autopsied	Total Estrone per Animal	Ave. Body Weight	Ave. Pituitary Weight	Ave. Lactogen per Pit.	Ave. Reece-Turner Units			Ave. % Increase in Lactogen per 100 gm. Body Weight
							Per pit.	Lactogen per mg. pit. tissue	Per 100 gm. bd. wt.	
			I. U.	gm.	mg.	I. U.				
A, Normal controls	M, 11	Unoperated	None	337	9.86	.138	3.06	.31	.90	
B, Adrenalectomized	M, 4	3	1200	333	12.97	.398	8.87	.68	2.66	195
C, Adrenalectomized	M, 2	4	1800	348	12.25	.386	8.62	.70	2.47	174
D, Adrenalectomized	M, 2	6	3000	386	16.22	.754	16.75	1.03	4.34	382
E, Adrenalectomized	F, 5	1–6	None	414	14.99	.270	6.00	.40	1.45	
F, Adrenalectomized	F, 5	1–6	600–3000	432	14.40	.450	10.00	.69	2.31	59

Adrenalectomy during advanced pregnancy in rats did not prevent the rise in the lactogen content of the pituitary following parturition (table 3). Reece and Turner previously found that the intact albino rat had 2.90 R.-T.U. of lactogen per pituitary on the 12th day of pregnancy, 3.50 R.-T.U. on the 19th day of pregnancy, and 7.72 R.-T.U. at 48 hours after parturition (18). On the whole, our assay results agree with the above data. The average lactogen content per pituitary in the albino rats during advanced pregnancy was 4.05 R.-T.U., while at 48 hours postpartum it rose to 8.25 R.-T.U. in the intact rats and to 8.00 R.-T.U. in the adrenalectomized rats. Both the intact and adrenalectomized mothers in the group of white-hooded rats contained about the same amounts of lactogen in their pituitaries at 48 hours after parturition. Thus, adrenalectomy during pregnancy did not prevent the increased lactogenic content of the pituitary which normally follows parturition.

TABLE 3. EFFECT OF ADRENALECTOMY ON POSTPARTUM RISE IN LACTOGEN CONTENT OF PITUITARIES OF FEMALE RATS

Group, Treatment	Strain	Stage at Autopsy	No. of Rats	Ave. Body Wt. gm.	Ave. Pit. Wt., mg.	Average Lactogen per Pit. I.U.	Ave. Reece-Turner Units Lactogen			Average % Increase in Lactogen per 100 gm. Body Weight
							Per pit.	Per mg. pit. tissue	Per 100 gm. body weight	
A, Normal	Albino	Advanced pregnancy	6	255	11.73	.182	4.05	.33	1.58	
B, Normal	Albino	48 hr. postpartum	6	186	11.02	.371	8.25	.74	4.43	180.0
C, Adrenalectomized	Albino	48 hr. postpartum	10	174	11.07	.360	8.00	.72	4.59	190.5
D, Normal	Hooded	48 hr. postpartum	4	152	9.27	.304	6.75	.72	4.44	181.0
E, Adrenalectomized	Hooded	48 hr. postpartum	3	154	11.07	.281	6.25	.56	4.06	157.0
F, Adrenalectomized[1]	Hooded	48 hr. postpartum	3	156	9.43	.281	6.25	.66	4.00	153.0

[1] Group F, received 1% NaCl in drinking water and feed.

The intensity of lactation in the adrenalectomized mother rats at 48 hours after parturition was obviously below that of the intact parturient rats, as judged by a macroscopic examination of the mammary glands. Also, about 50 per cent of the young of the adrenalectomized rats were dead by the second day after parturition, while most of the young from the intact rats were alive at this time. Examination of the stomachs of the living young from the adrenalectomized mothers revealed that milk was present, although apparently not in as great quantities as in the young of the intact mother rats.

DISCUSSION

The reduction in the lactogen content of the pituitaries of female albino rats following adrenalectomy may be due to one or several interacting factors: *a*) a direct inhibitory effect on the lactogen-producing cells of the anterior pituitary; *b*), cessation of the estrous cycles, withdrawing the normal estrogen stimulation to the pituitary; and *c*), a reduced food intake, resulting in a decreased production of hormones by the pituitary. There is no evidence at present which indicates that adrenalectomy directly affects the secretion of lactogen by the pituitary. It has been mentioned previously that adrenalectomy results primarily in a decrease of basophilic cells, and such cells are not presumed to secrete lactogenic hormone. Reece and Turner have shown, however, that a relationship does exist between the various phases of the estrous cycle and the lactogen content of the pituitary of albino rats (18). The highest amount of lactogen in the pituitary, 4.83 R.-T.U., was found during estrus, while the lowest amount of lactogen in the pituitary, 3.36 R.-T.U., was found at metestrus.

Ovariectomized albino rats contained 2.70 to 3.40 R.-T.U. of lactogen per pituitary, which is comparable to the level found at metestrus. Inasmuch as the estrous cycles cease in the rat after adrenalectomy, resulting in a condition comparable to metestrus or castration, it is not surprising that the lactogen content of the pituitary is reduced. The step-by-step course of events following adrenalectomy could thus be as follows:

Removal of the adrenals → Decreased secretion of gonadotropin by the anterior pituitary → Reduced stimulation of follicular growth in ovaries → Little or no estrogen to stimulate lactogen production in anterior pituitary → Reduced lactogen content in anterior pituitary

Although the above explanation for the reduced lactogen content in the pituitary of adrenalectomized rats seems plausible, the third possibility, a lowered food intake, may also be of some importance. The work of a number of investigators has shown that undernutrition results in an atrophy of the endocrine glands, which simulates the condition following hypophysectomy (24, 25). The available evidence indicates that this atrophy of the endocrine glands is due to a reduced secretory activity of the anterior pituitary, since upon the administration of anterior pituitary extracts these organs can be returned to normal (26–28). Therefore, the reduced food intake which follows adrenalectomy in the rat may be directly responsible for the reduced lactogen content in the pituitary, or indirectly, by reducing the gonadotropic secretion of the pituitary, it could set up the same train of events as pictured above.

It seems probable, in the albino rat at least, that adrenalectomy may also affect the ability of the pituitary to respond to estrogen. The increase in the lactogen content of the pituitaries of intact rats treated with estrogen was about 50 per cent greater than that obtained in the adrenalectomized rats. Since adrenalectomy, and the undernutrition which follows it, both affect the functional capacity of the anterior pituitary, this might account for the apparently subnormal stimulation effected by estrogen.

The fact that the postpartum lactogen content of the pituitaries of female rats adrenalectomized during advanced pregnancy increased to the same extent as in intact parturient rats, leads to the conclusion that the adrenals are not essential for the postpartum rise in lactogenic hormone. The ability of progesterone to substitute to some extent for the adrenal cortex during pregnancy may be of some importance in this connection. It is clear, however, that the inability of rats adrenalectomized during pregnancy to provide sufficient milk after parturition is not due to a deficiency of lactogenic hormone. This receives support from the work of Gaunt and Tobin (29) and Nelson and Gaunt (30, 31) who showed that adrenalectomized rats and guinea pigs cannot be induced to lactate by the administration of lactogenic hormone or anterior pituitary extracts. This would seem to exclude the possibility that the failure of lactation following adrenalectomy is due to a reduced secretion of anterior pituitary hormones. There is no conclusive evidence, however, that the adrenal cortex secretes a specific lactation factor, such as has been postulated by Hartman and his coworkers (32, 33). Gaunt has recently shown that a number of steroid hormones, including Kendall's compound *E* and dehydrocorticosterone, will maintain lactation in rats adrenalectomized after parturition (34). It is the opinion of the writers that the important upsets in mineral, water and carbohydrate metabolism which are known to arise as a result of adrenalectomy, are in themselves sufficient to account for the failure of lactation following such an operation.

SUMMARY

The effect of adrenalectomy on the lactogen content of the pituitary was determined in two groups of female albino rats totaling 33 animals. There was a reduc-

tion of 27.5 and 25.7 per cent, respectively, in hormone content on a body-weight basis, as compared with normal intact rats of the same species. This reduction in pituitary lactogen following adrenalectomy may be due to *a*), a direct inhibitory effect on the pituitary *b*), suspension of the estrous cycles, or *c*), reduction in food intake.

The administration of 1000 I.U. of estrone to 10 intact female albino rats increased the average lactogen content of the pituitary by 216.9 per cent on a body-weight basis, while the same amount of estrone injected into 14 adrenalectomized rats increased the average lactogen content of the pituitary by only 104.5 per cent. Male and female guinea pigs which were adrenalectomized and received estrogen, showed increases in pituitary lactogen equal to those obtained in intact guinea pigs. However, inasmuch as most of these guinea pigs survived but a few days after adrenalectomy, it is impossible to conclude that the full effects of adrenalectomy were operative on the pituitary.

Adrenalectomy of female albino and white-hooded rats during the last week of pregnancy did not prevent the postpartum rise in the lactogen content of the pituitary, which was found to be equal to that present in intact rats at 48 hours after parturition. Milk was present in the mammary glands of the adrenalectomized mother rats and also in the stomachs of their living young, but not in amounts equaling that found in intact rats and their offspring. It is concluded, therefore, that the failure of rats adrenalectomized during pregnancy to lactate sufficiently following parturition is not due to a deficiency of lactogenic hormone in the pituitary.

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MAMMARY GLAND GROWTH IN HYPOPHYSECTOMIZED CASTRATED GUINEA PIGS[1,2]

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A REVIEW of the recent literature reveals that the mechanism of growth of the mammary glands has been attributed to a number of factors (for review of literature, 1). Although the majority of evidence establishes quite conclusively that the anterior hypophysis exercises a controlling influence upon the mammary glands, there is some disagreement among investigators as to the rôle played by the anterior hypophysis in mammary growth. On the basis of an extensive series of experiments in a variety of hypophysectomized animals, it is suggested that the initiation and maintenance of mammary growth is under the control of the 'mammogen' secretion of the anterior hypophysis (2–6). Similar evidence has been interpreted by others to indicate that anterior hypophyseal secretions or certain extracts of the anterior hypophysis, especially those containing growth-promoting activity, are essential for mammary growth stimulation by estrogens (7, 8, 9). Estrogen alone was ineffective in stimulating mammary growth in several species of hypophysectomized animals (10, 13). It has also been suggested (14) either that estrogen facilitates the action of the mammogenic factor contained in the growth hormone preparation or the growth hormone with its mammogen factor facilitates and permits the action of estrogen on the mammary glands.

In view of these conflicting hypotheses this work was undertaken in order to determine whether *a*), an anterior hypophyseal extract free of all established anterior hypophyseal hormones can by itself induce mammary growth in hypophysectomized-castrated guinea pigs or *b*) whether any other established anterior hypophyseal hormone, including the lactogenic hormone, by itself or when in combination with steroid hormones, would cause growth of the mammary glands.

MATERIALS AND TECHNIQUE

In the first series of experiments the choice of donors of anterior hypophyseal glands were adult male and female guinea pigs. These pituitaries were extracted or used as implants. The mammogenic material was prepared from these glands by a slight modification of the extraction method described by Lewis and Turner (15). The fresh anterior hypophyseal tissue was extracted with several volumes of a hot mixture consisting of 8 parts of absolute ethyl alcohol and 2 parts of ether (U.S.P.) for 2 hours or more. The alcohol-ether solution was then filtered while hot and evaporated to dryness under reduced pressure. The material having mammogenic activity is

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[2] Read at the twenty-sixth annual meeting of the Association for the Study of Internal Secretions, Atlantic City, N.J., June 9, 1942.

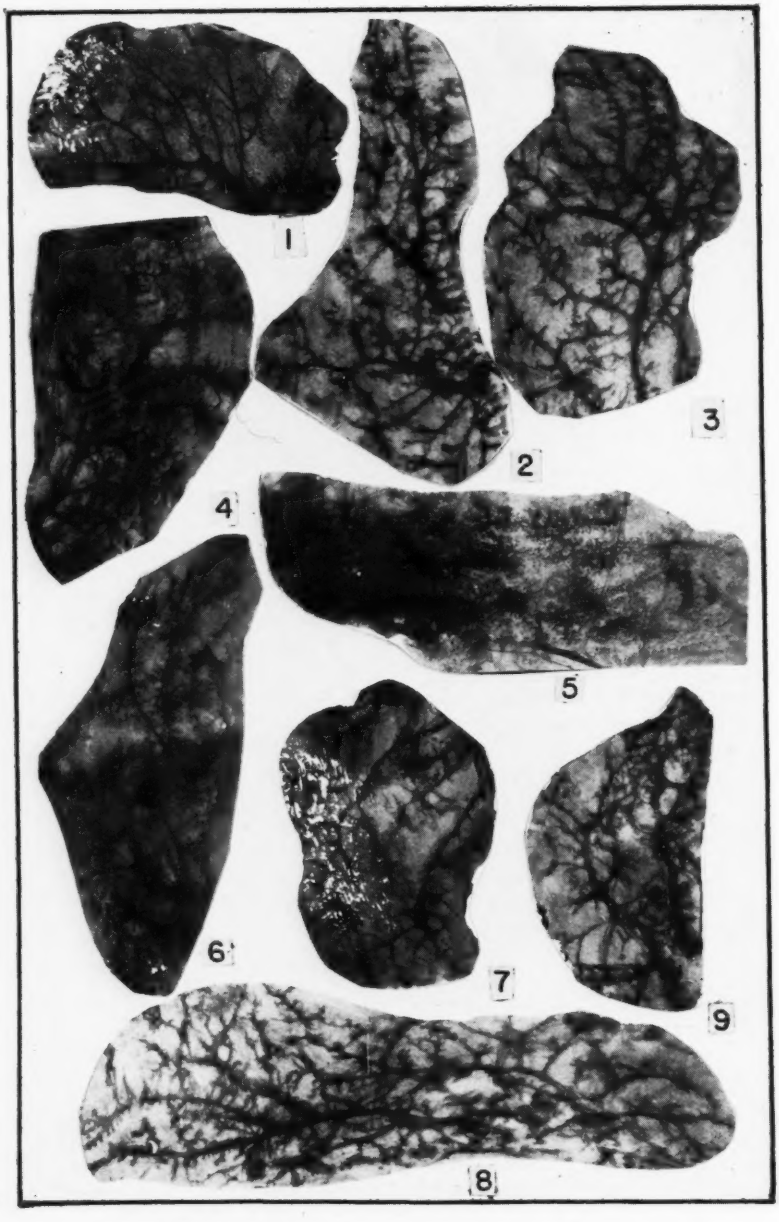

Fig. 1–9. MAMMARY GLANDS OF HYPOPHYSECTOMIZED CASTRATED GUINEA PIGS

Fig. 1. Mammary gland of a 60-day hypophysectomized-castrated female guinea pig (*1226B*) removed by biopsy at the time of the initiation of treatment with fresh anterior hypophyseal glands of adult female guinea pigs. Note the naked duct system characteristic of those of hypophysectomized guinea pigs of either sex. (×3).

contained in the resulting residue. The residue was recovered by dissolving it in ether and sesame oil and then removing the ether by evaporation in a warm water bath.

In the second and third series of experiments, the materials used were a relatively pure preparation of anterior hypophyseal hormone containing high lactogenic activity[3] and steroid hormones, including estrone, progesterone and desoxycorticosterone.[4]

The recipients were hypophysectomized male and female guinea pigs of a highly inbred strain[5] and weighing not more than 200 gm. at the beginning of treatment. Hypophysectomy and castration of the guinea pigs was performed at the time of weaning when the animals weighed from 150 to 200 gm. Treatment periods in all of the operated animals were usually from 10 to 15 days, beginning from 60 to 100 days after operation. In most instances, one mammary gland or a biopsy specimen was removed immediately preceding the initiation of treatment, for a control. The administration of hormone or hormone-containing materials was made once daily. The completeness of hypophysectomy was checked by a careful examination of the sella turcica under a high power binocular microscope. The growth of the mammary glands was determined by study of the whole mounts of stained and dissected glands.

EXPERIMENTAL RESULTS

The first purpose was to determine the minimum effective dose of fresh anterior hypophyseal gland tissue of guinea pigs for mammary growth, and whether it would be possible to demonstrate similar activity in an alcohol-ether soluble (mammogen) extract from an equivalent amount of tissue. Ten hypophysectomized-castrated male and female guinea pigs were each given from 20 to 50 mg. of fresh anterior hypophyseal gland tissue either by subcutaneous implants (2 animals) or by injection as a saline suspension, apportioned over a period of 10 days. The mammary glands of all of the test guinea pigs showed varying degrees of growth stimulation of the duct system after the various treatments (fig. 1, 2, 3). Ten similar guinea pigs were each in-

[3] The lactogenic hormone was kindly supplied by Dr. S. R. Hall, U. S. Bureau of Dairy Industry.

[4] Estrone, progesterone and desoxycorticosterone were supplied by the Schering Corp., Bloomfield, N.J., through the courtesy of Dr. E. Schwenk.

[5] The writer wishes to express his appreciation to Dr. O. N. Eaton, U. S. Bureau of Animal Industry, who supplied the guinea pigs for this study.

Fig. 2. Mammary gland from the same animal (fig. 1), but after receiving 50 mg. of fresh anterior-hypophyseal gland tissue of adult female guinea pigs implanted at intervals of one day over a period of 10 days. (×3).

Fig. 3. Mammary gland of a 60-day hypophysectomized-castrated female guinea pig (*1226C*) which received 50 mg. of fresh anterior hypophyseal gland of adult male guinea pigs in saline suspension, similarly apportioned over a period of 10 days. (×3).

Fig. 4. Mammary gland of a 100 days hypophysectomized castrated male guinea pig (*2742C*) which receives mammogenic extract (alcohol-ether extract) from 50 mg. of fresh anterior hypophyseal glands of adult female guinea pigs, similarly apportioned over a period of 10 days. (×3).

Fig. 5. Mammary gland of a 60-day hypophysectomized-castrated female guinea pig (*2742B*) which received mammogenic extract from 50 mg. of fresh anterior hypophyseal glands of adult female guinea pigs apportioned over a period of 10 days. (×3).

Fig. 6. Mammary gland of a 60-day hypophysectomized-castrated female guinea pig (*1242C*) which received mammogenic extract from 20 mg. of fresh anterior hypophyseal glands of adult female guinea pigs apportioned over a period of 10 days. (×3).

Fig. 7. Mammary gland of a 100-day hypophysectomized-castrated female guinea pig (*1242B*) which received 100 mg. of a lactogenic hormone preparation and 75 I.U. of estrone simultaneously, apportioned over a period of 10 days.

Fig. 8. Mammary gland of a 100-day hypophysectomized-castrated female guinea pig (*1226A*) which received 250 mg. of the same lactogenic hormone above (fig. 7) and 75 I.U. estrone, simultaneously, apportioned over a period of 15 days.

Fig. 9. Mammary gland of 60-day hypophysectomized-castrated female guinea pig (*21042*) which received 250 mg. of the residue of the lactogenic hormone after extraction with alcohol and ether, and 75 I.U. of estrone, simultaneously, apportioned over a period of 15 days. (×3).

jected with 'mammogenic' extract prepared from 20 to 50 mg. of fresh anterior hypophyseal glands in sesame oil, similarly apportioned in 10 daily doses. This resulted in a growth of the mammary glands, equaling that of early pregnancy (fig. 4, 5, 6). The extract produced no histological evidence of reparative effect upon the atrophied thyroid and adrenals of the test animals; there was no body growth-promoting effect (table 1) and, in the females, no cornification of the vaginal epithelium.

Seven other guinea pigs were each injected with a saline suspension of the residue after the alcohol-ether extraction in amounts equivalent to (3 animals) and up to 5 times (4 animals) the 20-mg. minimum effective dose level of fresh anterior hypophyseal glands apportioned over a period of 10 days. The mammary glands of these animals showed no evidence of growth stimulation after the treatments.

In view of the reports of mammary growth resulting from lactogenic hormone and estrone administration in hypophysectomized mice (16) and rabbits (17) a study was initiated in order to determine whether the administration of lactogenic hormone alone or in combination with estrone, progesterone or desoxycorticosterone would cause mammary growth in hypophysectomized guinea pigs.

Eight hypophysectomized-castrated guinea pigs were selected and divided into 4 experimental groups. The animals in all experimental groups were each given 100 mg. of lactogenic hormone; and, in addition, those of the second, third and fourth groups were given 75 I.U. of estrone, 50 mg. of progesterone or 50 mg. of desoxycorticosterone, respectively, as a total dose during a period of 10 days. In none of these 8 cases was there any evidence of mammary growth (table 1). The mammary glands presented the usual picture of a reduced naked duct system characteristic of those of hypophysectomized animals (fig. 7). Three similar guinea pigs were injected with 250 mg. of lactogenic hormone and 2 others with similar amounts of lactogenic hormone and 75 I.U. of estrogen over a period of 15 days. The mammary glands of animals in the former group showed a slight growth of the duct system at the end of the treatment. The duct growth response was improved in animals which received 75 I.U. of estrone in addition to the lactogenic hormone (fig. 8).

In order to determine whether the response noted above was due to the effect of mammogenic factor(s) in the lactogenic hormone or whether it is due to some other factor(s) in the lactogenic hormone which is capable of acting synergistically with estrogen to induce mammary growth, the lactogenic hormone used was extracted with the hot alcohol-and-ether mixture in a manner similar to that employed for the fresh anterior hypophyseal glands. Two hypophysectomized-castrated guinea pigs which were injected with 250 mg. of the alcohol-ether extracted residue and 2 others injected with similar amounts of lactogenic material and 75 I.U. of estrone showed no evidence of mammary growth after 15 days (fig. 9). The original, as well as the alcohol-ether extracted, lactogenic hormone preparation was tested for and found to have, in addition to its lactogenic property, appreciable growth, thyrotropic, gonadotropic and adrenotropic activities.

DISCUSSION

The evidence here presented confirms previous conclusions that mammary growth is under the direct control of the 'mammogen' secretion of the anterior hypophysis. A preparation obtained by extracting fresh anterior hypophyseal tissue of male or female guinea pigs with a hot mixture of alcohol and ether was found to have mammogenic potency in hypophysectomized-castrated guinea pigs of either sex. Of pertinent interest in this regard is the fact that the extract does not possess lactogenic, thyrotropic, adrenotropic, gonadotropic and growth activities. Lewis and Turner (18) reported similar activity of extract made from pituitaries of cattle. These authors

concluded that mammogen is soluble in lipoid solvents. Greep and Stavely (19), however, report that they were unable to show that lipoid solvents would remove any fraction from anterior hypophyseal gland tissue which would induce mammary growth in hypophysectomized rats.

Much evidence has been presented suggesting that the growth hormone, or a preparation possessing growth-promoting activity may supply the necessary substance(s) which enables the mammary glands to respond to estrogen. More recently

TABLE 1. EFFECT OF FRESH ANTERIOR HYPOPHYSEAL TISSUE OR EXTRACTS AND STEROID HORMONES ON MAMMARY GLAND GROWTH IN HYPOPHYSECTOMIZED-CASTRATED GUINEA PIGS

Experimental Treatment	Av. Change in Body Weight gm.	No. of Animals	Results
Experiment 1			
20–50 mg. fresh anterior hypophyseal tissue of male or female guinea pigs in 10 days			
a, by implants	+ 8	2	Very slight duct growth
b, by injections in saline suspension	+18	8	Extensive duct growth
Mammogenic (alcohol-ether) extract equivalent to 20–50 mg. fresh anterior hypophyseal gland tissue	− 6	10	Extensive duct and lobule-alveolar growth
A saline suspension alcohol-ether extracted anterior hypophyseal gland tissue			
20–50 mg. dose level in 10 days	0	3	No duct growth
100 mg. dose level in 10 days	− 9	4	No duct growth
Experiment 2			
100 mg. lactogenic hormone in 10 days	+12	2	No duct growth
100 mg. l.h.[1]+75 I.U. estrone in 10 days	+ 7	2	No duct growth
100 mg. l.h.[1]+50 mg. progesterone in 10 days	− 6	2	No duct growth
100 mg. l.h.[1]+50 mg. desoxycorticosterone in 10 days	−10	2	No duct growth
250 mg. lactogenic hormone in 15 days	+24	3	Slight duct growth
250 mg. l.h.[1]+75 I.U. estrone in 15 days	+ 8	2	Extensive duct growth.
Experiment 3			
250 mg. alcohol-ether treated lactogenic hormone in 15 days	+ 5	2	No duct growth
250 mg. l.h.[1]+75 I.U. of estrone in 15 days	+ 6	2	No duct growth

[1] l.h., lactogenic hormone

Gardner and White (20) reported mammary growth in hypophysectomized male mice treated simulataneously with prolactin and estrogen. Lyons (21) reported similar observations in hypophysectomized rabbits. In the present series of experiments a relatively purified preparation having, in addition to a high lactogenic activity and appreciable growth (table 1), thyrotropic, adrenotropic, and gonadotropic activities, also showed slight mammogenic activity in hypophysectomized-castrated guinea pigs. This activity was improved when estrogen was administered simultaneously with the lactogenic hormone.

Further experiments have shown that the mammogenic activity of the lactogenic hormone was no longer demonstrable after extraction with hot alcohol and ether, either with or without estrogen. Whether or not the hormone fraction(s) having mammogenic activity was removed or rendered inactive by the solvent remains to be determined. In view of the fact, however, that the other identified activities were not removed or destroyed by the solvent, these results were interpreted as indicating that mammary growth was manifested by a mammogen fraction(s) in the lactogenic hormone.

SUMMARY

Growth of the duct system of the mammary glands of hypophysectomized-castrated male and female guinea pigs has been induced either by implanting or injecting macerated fresh anterior hypophyseal gland tissue of adult male or female guinea pigs. The minimal effective dose of fresh anterior hypophyseal tissue was found to be approximately 20 mg. It was possible to make an alcohol-ether extract from the same weight of fresh tissue which was even more highly active than the fresh tissue. This and similar extracts which induce mammary growth, equaling that of early pregnancy, were without demonstrable activities of all the established anterior hypophyseal hormones.

A slight mammary duct growth response was induced also in hypophysectomized-castrated animals by injections of a lactogenic hormone preparation. This response was improved when estrogen was administered simultaneously with the lactogenic hormone. The preparation possessed, in addition to its mammogenic activity, high lactogenic and appreciable growth, thyrotropic, adrenotropic and gonadotropic activities. Extraction with a hot alcohol-and-ether mixture rendered the preparation only mammogenically inactive. These experiments further the concept that anterior hypophyseal mammogen(s) is the direct agent of mammary growth in hypophysectomized-castrated guinea pigs.

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EFFECTS OF HORMONES ON ERYTHROPOIESIS IN THE HYPOPHYSECTOMIZED RAT

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THE ANEMIA which occurs in rats after hypophysectomy has been studied by several groups of investigators (1–8). At first this anemia was attributed directly to the lack of some pituitary fraction or hormone (1, 3, 4). More recently, however, it has been interpreted as a reflection of metabolic changes resulting from a deficiency of several hypophyseal principles (5, 6, 8). Thus Meyer and his colleagues (6) have shown that growth, thyrotropic, andrenotropic hormones, and thyroxine evoked hemopoietic activity in hypophysectomized rats; and we (8) have reported that pregnant mare serum administered to hypophysectomized male animals and testosterone propionate to hypophysectomized rats of either sex can restore the normal red cell counts.

Such results, while interesting, have complicated rather than clarified the problem. As a step in the elucidation of the problem, it appeared necessary to extend our knowledge of the anemia of hypophysectomized animals. Therefore, we have studied the response of the erythrocytic elements of hypophysectomized rats during treatment with adrenal cortical hormone, thyroid hormone, desoxycorticosterone acetate and prolactin.

Several papers in the past few years have indicated that the metabolic action of thyroid hormone may be augmented by androgens (9, 10) and depressed by estrogens (11–13). Because of the possibility that the effects upon the blood picture may be mediated through the thyroid, some results on oxygen consumption have been included.[1]

MATERIALS AND METHODS

To obtain results comparable with those secured with gonadotropic and sex hormones, we have adhered, in general, to the procedure previously followed (8). It was felt advisable to begin treatment several weeks after hypophysectomy in order to allow time for the development of a marked anemia. Male rats 150 to 230 gm. in weight were hypophysectomized in the same way as before (5) except that nembutal was used as the anesthetic. The hematological and histological techniques were also the same. In addition, some of the marrows were removed from the bone and fixed in Downey's fluid. The technique used to determine oxygen consumption was that of Tainter and Rytand (15).

Except where otherwise indicated, the hormones were injected subcutaneously.

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[1] We wish to thank Dr. Malvina Schweizer for supplying the metabolic data, obtained partly from observations on our animals and partly from her own records (14).

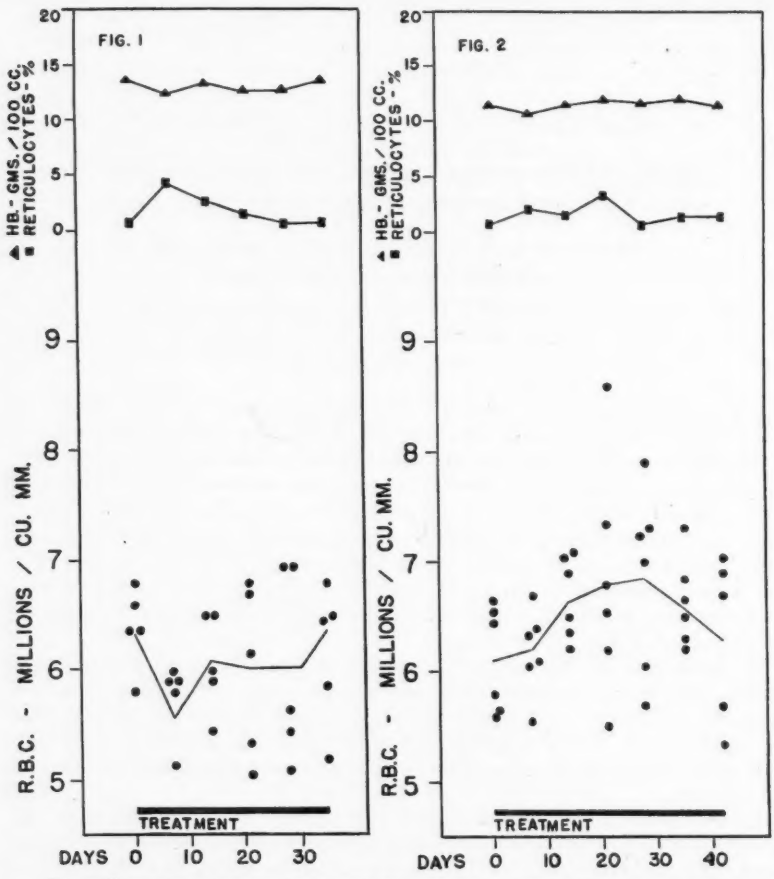


Fig. 1. Erythrocyte counts, mean hemoglobin determinations and mean reticulocyte percentages in hypophysectomized rats injected daily with 1–2 cc. of adrenal cortical extract, from the 6th week after operation.

Fig. 2. Erythrocyte counts, mean hemoglobin determinations and mean reticulocyte percentages in hypophysectomized rats injected daily with 1.0 mg. of desoxycorticosterone acetate from the 6th week after operation.

The preparations[2] included adrenal cortical extract, desoxycorticosterone acetate, prolactin, crystalline thyroxine[3], thyroid powder[4] and testosterone propionate.

RESULTS

Adrenal cortical hormone. Five hypophysectomized rats were injected with 1 to 2 cc. daily (in 3 doses) of adrenal cortical extract, for a period of 5 weeks, beginning with

[2] We are indebted to Dr. Erwin Schwenk, Schering Corp., Bloomfield, N. J., for generous supplies of testosterone propionate (Oreton), desoxycorticosterone acetate (Cortate), and prolactin (1 mg. equals 12 I.U.). We also wish to thank Dr. W. W. Swingle of Princeton University for the adrenal cortical hormone (1 cc. equals 50 gm. of tissue).

[3] The crystalline thyroxine was supplied by Roche-Organon Inc., Nutley, N. J.

[4] The thyroid powder was supplied by Armour and Co., Chicago, Ill.

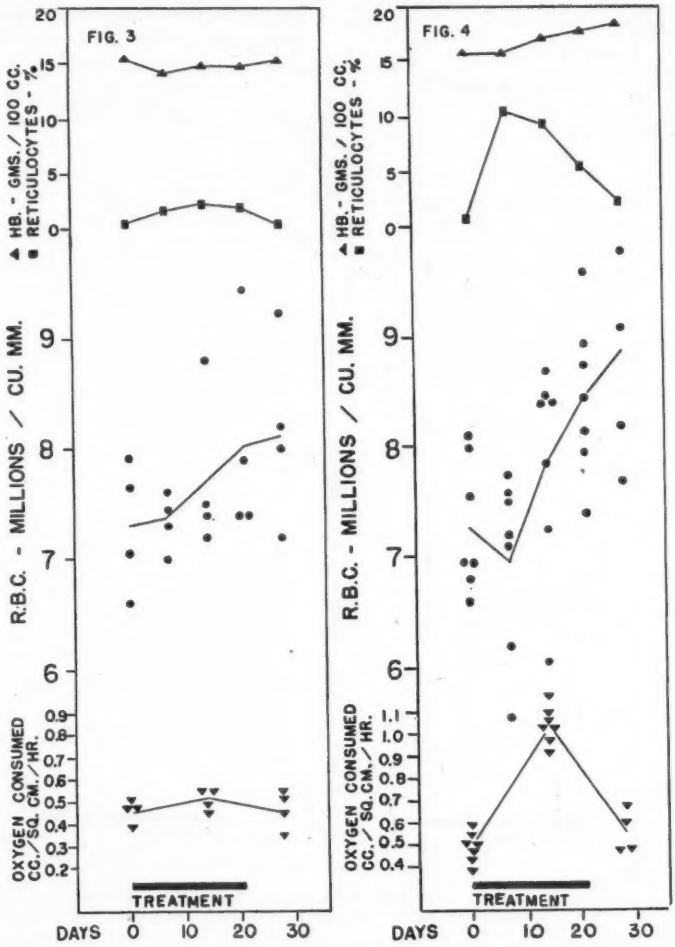


Fig. 3. ERYTHROCYTE COUNTS, MEAN HEMOGLOBIN DETERMINATIONS, MEAN RETICULOCYTE PERCENTAGES AND OXYGEN CONSUMPTION in hypophysectomized rats injected daily with 1.0 mg. of desoxycorticosterone acetate, from the 3rd week after operation.

Fig. 4. ERYTHROCYTE COUNTS, MEAN HEMOGLOBIN DETERMINATIONS, MEAN RETICULOCYTE PERCENTAGES AND OXYGEN CONSUMPTION in hypophysectomized rats injected daily with 1.0 mg. of desoxycorticosterone acetate and fed with a ration containing 8.3 mg. of thyroid powder per gm., from the 3rd week after operation.

the 6th week after operation. During this time the erythrocyte counts and hemoglobin determinations remained approximately at their initial levels, except for a transient drop in red cell number at the end of the first week (fig. 1). The reticulocytes showed a fairly definite rise at this time, then gradually declined to the low percentages characteristic of hypophysectomized rats. At autopsy, the adrenals, thyroids, gonads, and reproductive structures were found to be atrophied. Histological sections of these glands revealed no stimulation. The bone marrows of the treated animals were extensively vacuolated and although several areas of proliferating red cells were observed,

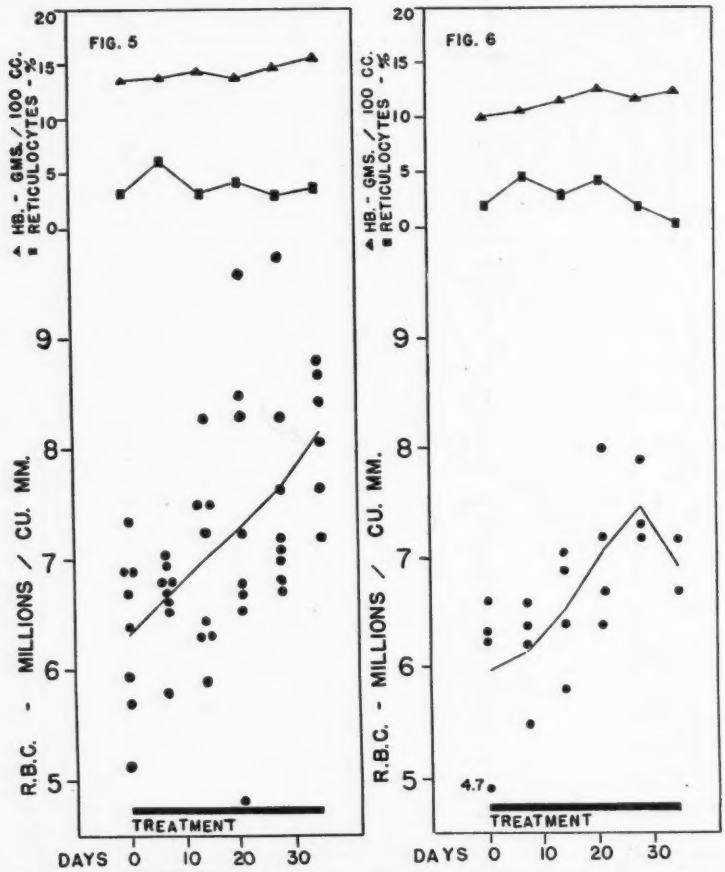


Fig. 5. Erythrocyte counts, mean hemoglobin determinations and mean reticulocyte percentages in hypophysectomized rats injected daily with 0.01–0.03 mg. of thyroxine, from the 6th week after operation.

Fig. 6. Erythrocyte counts, mean hemoglobin determinations and mean reticulocyte counts in hypophysectomized rats injected daily with 0.5–1.5 mg. of prolactin, from the 6th week after operation.

the picture, in general, resembled that seen in untreated hypophysectomized rats (fig. 8, 9). In another group of 4 hypophysectomized male rats injected daily with adrenal cortical extract, no change in oxygen consumption was observed (14).

Desoxycorticosterone acetate. Six animals (*group 1*), hypophysectomized 1.5 months previously, were injected daily with 1.0 mg. of desoxycorticosterone acetate, for a period of 40 days. The erythrocyte count[5] increased from 6.1 ± 0.2 to 6.9 ± 0.4 million per cu. mm. in the 4th week, but this apparent gain had almost disappeared in the 6th week (fig. 2). The mean hemoglobin value remained unchanged during treatment, and the reticulocyte response was feeble. The weights of the gonads, adrenals and thyroids of these animals were the same as those of untreated hypophysectomized

[5] Mean values of counts are given with the standard error.

animals. Histologically, these organs showed no signs of stimulation. Vacuolization of the bone marrow was marked. Erythroid elements were considerably less concentrated than in normal marrows (fig. 10).

Four additional animals (*group 2*) which had been hypophysectomized for only 3 weeks, were also injected daily with 1.0 mg. of desoxycorticosterone acetate (fig. 3). Their initial count was 7.3 ± 0.3 million erythrocytes per cu. mm. (about midway between that of unoperated animals and that of long-time hypophysectomized ones); one month later the count was 8.2 ± 0.5 million. The latter figure should be compared with the counts of untreated rats 7 weeks after hypophysectomy, which usually lie between 5 and 6 million per cu. mm. (5). The hemoglobin values of this group, however, did not rise, and the reticulocyte increase was negligible. Determinations on these and other hypophysectomized rats injected with desoxycorticosterone acetate indicated that there was no change in oxygen consumption during treatment.

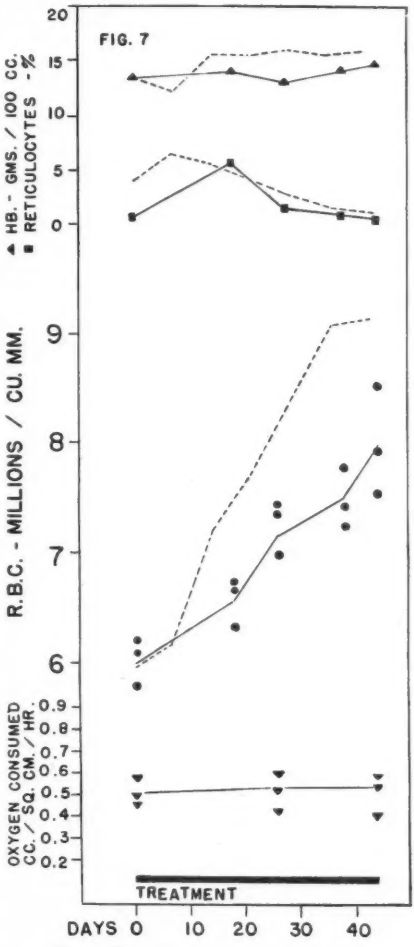
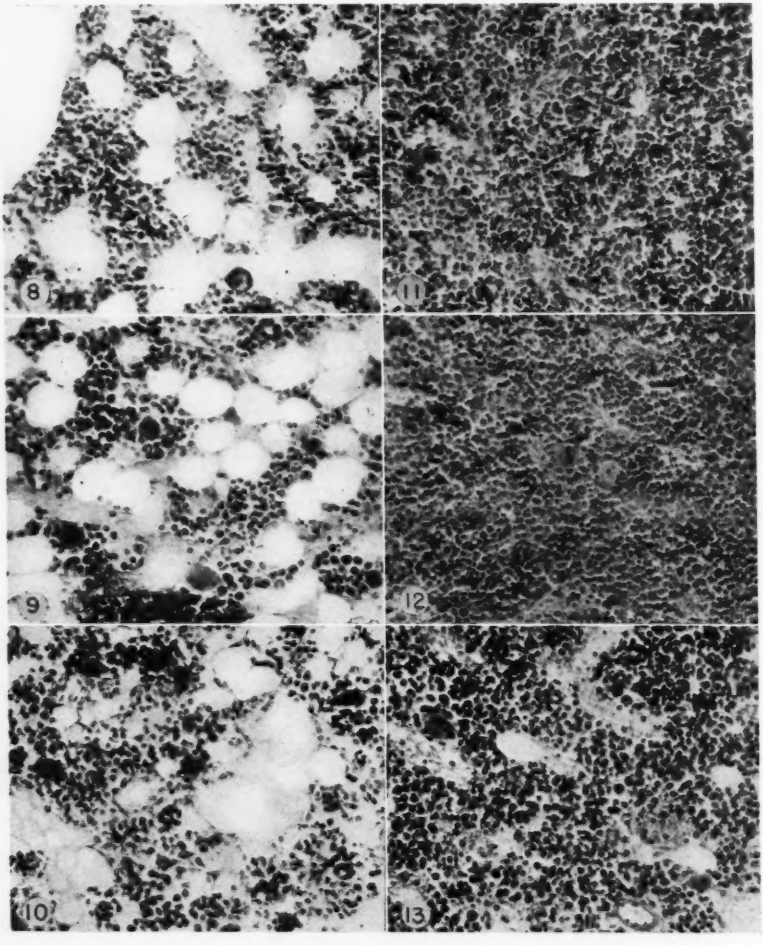


Fig. 7. ERYTHROCYTE COUNTS, MEAN HEMOGLOBIN DETERMINATIONS, MEAN RETICULOCYTE COUNTS AND OXYGEN CONSUMPTION in rats injected daily with 2.0 mg. of testosterone propionate, from the 6th week after operation. The dotted lines represent mean determinations for a similar group of 5 animals, injected daily with 1.0 mg. of testosterone.

Thyroid powder and desoxycorticosterone acetate. Figure 4 shows the data for 7 hypophysectomized rats injected daily with 1.0 mg. of desoxycorticosterone acetate and fed with a ration containing 8.3 mg. of thyroid powder per gm., beginning with the 3rd week after operation. The mean red cell count rose from 7.3 ± 0.3 to 8.5 ± 0.3 million per cu. mm. in 3 weeks' time. Hemoglobin values also increased, approximately to normal. The reticulocytes increased sharply from 0.9 to 10.6 per cent during the first week, then declined gradually until the end of the experiment. Oxygen consumption was found to have doubled after 2 weeks of treatment, but dropped back to the initial range a week after treatment was discontinued. The bone marrows of these animals showed considerable repair. Vacuoles were almost completely absent. Actively dividing erythrogenic elements were found in compact nests scattered throughout the entire marrow (fig. 11).

Crystalline thyroxine. A similar picture was presented by a group of 8 hypophy-



SECTIONS THROUGH THE SAME REGIONS OF RIGHT FEMORAL BONE MARROWS FIXED IN BOUIN'S SOLUTION AND STAINED WITH HEMATOXYLIN-EOSIN.

Fig. 8. Untreated; 3 months after hypophysectomy showing marked vacuolization and diminution in the numbers of erythrogenic elements.

Fig. 9. Hypophysectomized 6 weeks and then injected daily for 5 weeks with 1-2 cc. of adrenal cortical extract. General hypoplasia is about the same as in figure 8.

Fig. 10. Hypophysectomized 6 weeks and then injected daily for 40 days with 1.0 mg. of desoxycorticosterone acetate. Hypoplasia is marked.

Fig. 11. Hypophysectomized 3 weeks and then fed 8.3 mg. of thyroid powder (per gm. ration) and injected with 1.0 mg. desoxycorticosterone acetate daily for 40 days. Hypoplasia is no longer apparent. Vacuoles have become replaced by actively proliferating erythrogenic cells.

Fig. 12. Hypophysectomized 6 weeks and then injected daily for 6 weeks with 0.01 to 0.03 mg. of thyroxine. Repair is considerable. Newly formed erythrogenic elements and megakaryocytes are numerous.

Fig. 13. Hypophysectomized 6 weeks and then injected daily for 5 weeks with 0.5-1.5 mg. of prolactin. Vacuolization is not so marked as in figure 8. Some repair of the post-hypophysectomy hypoplasia is evident.

sectomized rats injected daily with 0.01 to 0.03 mg. of crystalline thyroxine beginning with the 6th week after operation. During 5 weeks of treatment the erythrocyte count increased at a uniform rate, from 6.4 ± 0.3 to 8.2 ± 0.3 million per cu. mm. (fig. 5). Hemoglobin values also rose, but the percentage increase was only half as great. The increase in reticulocyte percentage was smaller than that seen in animals treated with thyroid and desoxycorticosterone acetate, several rats failing to show any rise while in one rat the percentage increased ten-fold. Body weight declined somewhat in this group, and the organ weights remained low. Histologically, the adrenals, testes, and thyroids were atrophic. The bone marrows presented an appearance of complete repair. Erythroid elements showed marked proliferation. Increased vascu-

TABLE 1. BLOOD CHANGES IN HYPOPHYSECTOMIZED RATS TREATED WITH VARIOUS HORMONES

Treatment	Per Cent of Increase[1] Red Blood Cells	Hb.	Highest Reticulocyte Count,[2] %	Condition of Bone Marrow
Adrenal cortical extract	0	0	4.1	Hypoplastic
DCA[3] (group 1)	13	0	3.3	Hypoplastic
(group 2)	12	0	2.1	(No data)
DCA and thyroid	22	17	10.7	Complete repair
Thyroxine	28	15	6.1	Complete repair
Prolactin	25	16	4.7	Moderate repair
Testosterone[4]				
(group 1)[5]	51	17	6.1	Complete repair
(group 2)	33	12	5.5	Almost complete repair
PMS (in males)[5]	31	19	(No change)	Complete repair

[1] For erythrocytes and hemoglobin the percentage increase of the highest mean weekly determination over the initial mean determination is given; this usually corresponds to the fourth or fifth week.

[2] For reticulocytes the highest weekly mean count during the period of treatment is given; this usually corresponds to the first or second week.

[3] *Group 1* of the rats treated with desoxycorticosterone acetate had been hypophysectomized 6 weeks prior to treatment, while *group 2* had been operated only 3 weeks before treatment began.

[4] *Group 1* of the testosterone-treated animals was injected with 1.0 mg. daily, while *group 2* was injected daily with 2.0 mg.

[5] Data previously published (8).

larity of the marrow and increased numbers of megakaryocytes were also observed (fig. 12). Metabolism was restored to normal in 4 other animals similarly treated.

Prolactin. Four 6-week hypophysectomized rats injected daily with 0.5 to 1.5 mg. of prolactin showed moderate gains in erythrocyte counts and hemoglobin levels; and reticulocyte values were generally higher during treatment than before (fig. 6). Weight and histology of the adrenals, testes and thyroids were unchanged. In the bone marrow sections, erythrogenic elements appeared to have increased in number, and the marked vacuolization usually found in marrows of hypophysectomized rats was not to be seen (fig. 13). The extent of the repair, however, was not so great as that seen in the thyroid or thyroxine-treated groups.

Testosterone propionate. This experiment was made to determine whether the erythropoietic effects of testosterone previously reported (8) were accompanied by any change in oxygen consumption. Three hypophysectomized rats injected daily with 2.0 mg. of testosterone, starting 6 weeks after the operation, did not reveal increased oxygen intake, although their mean erythrocyte count rose from 6 to 8 million per cu. mm. (fig. 7). Despite considerable reticulocytosis, the rise in the numbers of red blood cells and hemoglobin level in these 3 animals was distinctly less

rapid than that previously seen in rats injected daily with 1.0 mg. of the same hormone (dotted lines in fig. 7).

A summary of pertinent data from this and a previous paper is given in table 1.

DISCUSSION

The changes in the red cell count induced by hormones, as presented in this paper, may be attributed either to an altered rate of erythrocyte production or to hemoconcentration. In this regard we have already presented evidence that the changes in erythrocyte number after hypophysectomy and during substitution therapy are, on the whole, due to effects on blood cell production (5, 8). Histologically there is consistent evidence of bone marrow hypoplasia after hypophysectomy, and marrow repair when hypophysectomized rats are treated with testosterone, pregnant mare serum (in the male), thyroxine, or prolactin; also with growth hormone (6) and some anterior pituitary preparations (3). Adrenal cortical extract and desoxycorticosterone acetate fail to repair the hypoplasia, although the latter substance seems to prevent the red cell count from falling to the long-time hypophysectomy level.

Additional evidence in favor of altered rates of erythropoiesis lies in the relationtionship between the red cell counts and the hemoglobin determinations. As seen from table 1, in every group in which there is an increase in erythrocyte number, this increase is proportionately greater than the increase in hemoglobin, *i.e.* 3 times greater in the groups injected with testosterone. The increase in red cell count, therefore, could not be attributed to hemoconcentration. It is much more likely that the difference in percentage increase is due to a release into the circulation of new cells of low hemoglobin content. It is interesting to note that animals made anemic by thyroidectomy respond similarly to thyroid administration, with polycythemia, while the hemoglobin level remains below normal (16).

Another criterion of erythrocyte production, upon which some workers have relied too much, is reticulocytosis. All of the treated groups listed in table 1 show increased numbers of reticulocytes. This includes the group injected with cortical extract, which displays no other sign of marrow activity. Overbeek (2) was able to evoke reticulocytosis in hypophysectomized rats with glucose and with olive oil; Meyer and coworkers (6) did the same with cevitamic acid and with bicarbonate of soda, without effect on the animals' anemia. A change in reticulocyte percentage alone therefore, cannot be regarded as a measure of red cell output by the bone marrow. We are inclined to regard reticulocytosis as important only when it precedes a rise in circulating erythrocytes, and when the reticulocyte percentage falls as the red blood cell count increases. This is the normal pattern of the reticulocyte response to hemopoietic agents (17, 18). The reticulocytes may give an especially false impression in experiments with hypophysectomized rats, as the reticulopenia of these animals may be interrupted suddenly and spontaneously; Overbeek (19) has observed this, and we have seen high reticulocyte counts in many animals more than 6 weeks following hypophysectomy, especially if the red cell count has fallen as low as 5 million per cu. mm.

Upon the basis of the criteria listed, a true bone marrow stimulation has been demonstrated in hypophysectomized animals treated with thyroxine, testosterone, and, to a lesser degree, prolactin. The effect of desoxycorticosterone acetate is difficult to interpret. Cortical hormone is clearly ineffective. This is in agreement with Meyer's (6) results which show that adrenotropic hormone does not prevent the post-hypophysectomy anemia.

The mode of action of these hormones represents another point of contention. It is possible that the hormones directly influence the production of red blood cells

in the marrow. The hypophyseal 'hemotropic' hormone of Flaks, et al. (3) would be such a fraction, but we have shown that thyroxine and testosterone are also effective in the hypophysectomized rat. Thus, no single hormone seems to be absolutely necessary for erythropoiesis, while several can act as stimulants. This fact may indicate that none is a direct stimulant, but that each can act through a channel, common to all, which may be a metabolic one. Thyroid, for example, might produce its effect on the blood picture by increasing somatic respiration, thus creating a greater demand for red cells. Pituitary fractions also might do this in the hypophysectomized rat (20, 21). Androgens (9, 10) and estrogens (11–13) modify the effectiveness of thyroid hormone. Many other details may be made to fit into the picture, if increased oxygen demand is assumed to be the basis of action of erythropoietic hormones. However, neither testosterone nor desoxycorticosterone acetate were found to stimulate the thyroid gland or to increase oxygen consumption in the hypophysectomized rat, although testosterone brought the cell count to normal and desoxycorticosterone acetate kept it above the long-time hypophysectomy level. Increased rate of oxygen consumption, therefore, does not seem to be the common metabolic channel.

Compatible with the idea of direct action but not a necessary corollary, is the possibility that anoxia, 'the fundamental erythrocytogenic stimulus,' causes the release of hormones to stimulate the bone marrow. This view would receive indirect support from the fact that the marrow of the hypophysectomized rat does not respond to low oxygen pressure, except when growth hormone is always administered (6). However, it is possible that the hypophysectomized rat, with its low basal metabolic rate, is able to meet its needs under low pressure by means other than increased erythropoiesis.

While it is not possible, at present, to explain the hemopoietic action of hormones, some of the lines along which further research should proceed are clear: further replacement in animals deprived of endocrine organs, with cytological study of the bone marrow; treatment of endocrine-deficient animals with various metabolic stimulants, known hemopoietic substances, and nutritive factors; and further attempts to find a common metabolic denominator by which hormone action can be linked with the respiratory function of blood.

SUMMARY

Thyroxine causes changes in the blood picture of hypophysectomized animals resembling the changes brought about by testosterone, *i.e.*, markedly increased erythrocyte number, slightly increased hemoglobin level, reticulocytosis, and repair of the bone marrow. Prolactin produces similar but less pronounced alterations in the blood picture. Desoxycorticosterone acetate maintains the red cell count and hemoglobin level above those of untreated hypophysectomized rats, without bringing about marrow repair. The thyroids of hypophysectomized animals treated with testosterone, prolactin and desoxycorticosterone acetate are like those of untreated hypophysectomized rats in weight and in histological appearance. There is no increase in oxygen consumption in hypophysectomized animals treated with desoxycorticosterone acetate, cortical hormone or testosterone. It is concluded that some hormones can act as erythropoietic agents without altering the rate of oxygen consumption, and that these effects, at least in the hypophysectomized rat, are not mediated through the thyroid gland.

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COMPARISON OF THE THERMOSTABILITY OF GROWTH AND KETOGENIC ACTIVITIES OF ANTERIOR PITUITARY EXTRACT

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THE PRECISE NUMBER of anterior pituitary hormones is not known. Proof of the separate identity of these substances is difficult because most of the anterior lobe principles cannot be obtained at present in pure form. Methods of study, therefore, remain indirect. For example, it is possible to subject an extract to chemical or physical treatment which will result in a preparation that has lost one hormonal activity but not another. The conclusion then follows that the two activities are not due to a single molecular entity. Complete removal of one activity and complete preservation of the other is not necessary in order to apply such a deduction inasmuch as this reasoning likewise applies to extracts in which a distinct change in the ratio of potencies of two activities has been produced. In previous experiments, Shipley and Long (1) showed by this method that growth and ketogenic activities could not be identified with the thyrotropic, adrenotropic, gonadotropic or lactogenic principles. That growth and ketogenic activities were themselves due to two separate principles could not be established, however, because these two activities ran so strikingly parallel during various fractionation procedures, that one was justified in suspecting that both effects were attributable to a single chemical entity. Black, Collip, and Thomson (2) prepared a growth extract which, after repeated adsorption on calcium phosphate, was considered to have lost its ketogenic properties. Collip, however, later stated that he knew of no growth preparation which was entirely free from ketogenic activity (3).

An anterior pituitary preparation which is high in growth and ketogenic activity, free from thyrotropic and gonadotropic activity, and low in prolactin has been previously described (1). This fraction is soluble in 80 per cent acetone at pH 3.5, and is relatively insoluble in aqueous solution near the neutral point. Further attempts to dissociate the growth and ketogenic properties of this fraction by treatment with graded concentrations of sodium sulfate at various values of pH have met with no success. Ultrafiltration of crude extract through graded cellophane filters likewise has not provided a means of separation of the two principles, although observations have been made on molecular size which will be presented in a separate report. Evidence for the separate identity of growth and ketogenic principles was ultimately obtained through observations on the relative stability of the two activities when an extract was heated at various hydrogen-ion concentrations. These latter experiments comprise the material of the present report.

METHODS AND RESULTS

An extract prepared according to the method of Burn and Ling (4) and derived from beef anterior pituitary was frozen; the gelatinous material that formed was re-

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moved by centrifugation. Portions of the extract were adjusted to pH 10.0, 7.5, 4.5, 2.0, and 1.0 with the final volume of each portion brought to twice that of the original. Each solution was placed in a 15 cc.-test tube of 15 mm. inside diameter and immersed in a boiling water-bath for 10 minutes. After prompt cooling of the solutions, the pH was readjusted to approximately 7.5. The original extract and heated preparations were satisfactorily preserved by storage in the frozen state at −15° C.

Ketogenic activity was determined by measurement of the rise in blood-acetone bodies produced in fasting rats after the injection of a measured dose of extract. The essential details of the method have been previously published (1). Except for those rats receiving the extract boiled at pH 10.0, the number of animals given each heated preparation varied from 20 to 28, and two dosage levels were used. Doses were chosen which gave responses within the 'straight line' portion of a standard assay curve. The standard curve was constructed from responses obtained at 8 different dosage levels in a total of 117 rats which had received the original, unheated extract (Burn and Ling).

Growth assays were performed on hypophysectomized rats weighing from 90 to 110 gm. The animals were observed for stationary weight over a period of 2 to 3 weeks both before and after injection. Extracts were administered intraperitoneally once a day for 6 days in at least two different dosages (except for preparation *B*-10) and here again at such a dose level as to produce a weight gain within the straight line portion of the logarithm-dose response curve. Some rats were used only once while others were injected a second time immediately after the first period. No loss of sensitivity was evident in the animals during a second assay period. This statement is based on a comparison of the response produced by a given extract in 11 unused rats with the response in 18 other rats which had already been subjected to one assay. Further use of the animals after two 6-day periods of assay was unsatisfactory as there was then commonly a noticeable decrease in reactivity. From 13 to 18 animals were used for the assay of each heated preparation (except *B*-10), while 33 animals received the original unheated extract.

Calculation of the relative potency of a heated preparation was by a graphical method. In figure 1 is reproduced a chart of the ketogenic response of 117 rats receiving graded doses of the standard extract. The curve is sigmoid, but for practical purposes the region between the 10 and 40-microgram doses may be considered a straight line. To the right of the standard curve, and opposite its straight portion, are

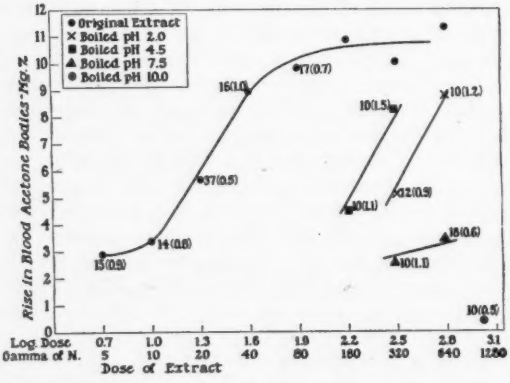

Fig. 1. Ketogenic response. The figures after the points on the chart represent the number in the group from which this average response was obtained. The numbers in parenthesis are standard error of the mean. Dosage is expressed both in micrograms of nitrogen and the logarithmic equivalent.

two lines representing the response to extract boiled at pH 4.5 and pH 2.0, respectively. The slope of these lines and that of the standard curve in this region are very nearly the same and may be assumed to be identical for purposes of assay. The potencies of the two boiled preparations in terms of the standard are equivalent to the reciprocal of the anti-logarithm of the horizontal distance between their assay line and the straight portion of the standard curve (measurement in logarithm-dose units). For extract boiled at pH 4.5, this distance is 0.98 which represents a potency of 1/10, and for extract boiled at pH 2.0, the distance is 1.22, and the potency 1/17. In terms of percentage of activity retained, the values are 10 and 6 per cent, respectively. The potency of the preparation boiled at pH 7.5 was determined in similar manner, but in this case the portion of the standard curve used for comparison was the lower segment between the 5 and 10-microgram doses. The calculated potency was 1/72 or 1.4 per cent retention. The extract boiled at pH 10 may be considered totally inactive. The response to 1000 μg. was only 0.4 mg. per cent while a series of 12 uninjected control animals showed a rise of 0.8 mg. per cent.

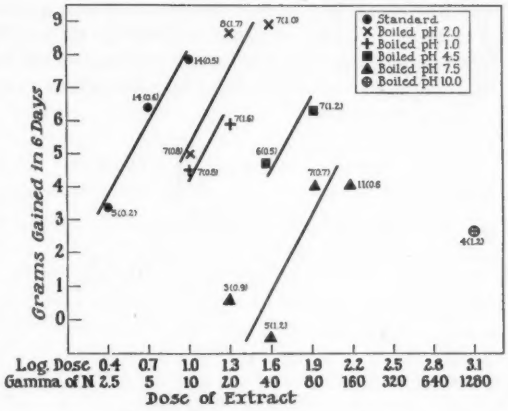
Standard
Boiled pH 2.0
Boiled pH 1.0
Boiled pH 4.5
Boiled pH 7.5
Boiled pH 10.0
Grams Gained in 6 Days
Log. Dose 0.4 0.7 1.0 1.3 1.6 1.9 2.2 2.5 2.8 3.1
Gamma of N 2.5 5 10 20 40 80 160 320 640 1280
Dose of Extract

FIG. 2. GROWTH RESPONSE CURVES.

The calculation of growth potency was similar to that described above and was performed with the help of the graph shown in figure 2.

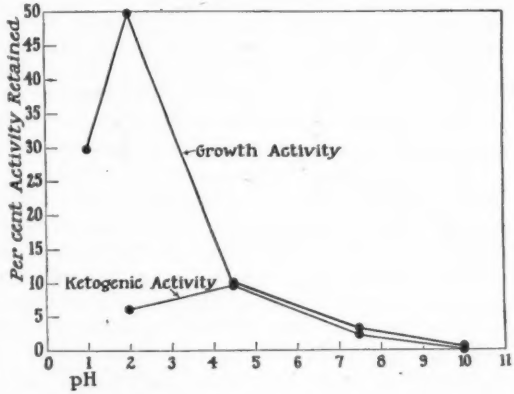

FIG. 3. COMPARISON OF THE GROWTH AND KETOGENIC ACTIVITY retained after heating the extract at various H-ion concentrations.

It may be observed from figure 3 that boiling at pH 10.0 resulted in practically a complete loss of both growth and ketogenic potency. Near the neutral point, some activity of each was retained but it was in the acid range that both activities exhibited their maximum thermal stability. The resistance of growth principle to destruction at pH 2.0, however, was much greater than that of the ketogenic. Thus, at this acidity the loss in growth-promoting power was only 50 per cent, as contrasted with a 94 per cent loss of ketogenic potency. The stability curve of ketogenic substance also varied from that of the growth principle, in that its peak was at

pH 4.5, while that of the latter was at 2.0. A substantial degree of growth activity was retained even after heating in the medium with an acidity as high as pH 1.0. Insufficient extract was available to assay this latter preparation for ketogenic activity.

The observation that heating at pH 2.0 reduces ketogenic activity to 1/17 and growth activity to only 1/2 strongly suggests that the two effects are not due to a single substance. The statistical error of these estimated potencies is obviously of extreme importance if one is to make any such deduction. The ratios of 1/17 and 1/2 which were obtained for the relative ketogenic and growth potencies of the extract heated at pH 2.0 may be submitted to statistical treatment for estimation of the error of an assay such as that described by Bülbring (5), Bliss and Marks (6), and Light, deBeer, and Cook (7). A somewhat similar method devised by Ross is contained in an article by Campbell (8). Bülbring's formula may be expressed thus:

$$\lambda = \frac{\sigma}{b}\sqrt{\frac{1}{n_1}+\frac{1}{n_2}}$$

Where λ is the standard error of the logarithm of the potency ratio, σ is the weighted mean standard deviation of the response within a number of single groups receiving a given dose of similar extracts, b is the slope of the mid-portion of the logarithm-dose response curve (which is assumed to be constant for both extracts), and n_1 and n_2 are the total number of animals receiving *extract 1* and *extract 2*, respectively.

The formula, as applied to the ketogenic potency ratio of 17 gives: Logarithm potency $\pm$ standard error $= 1.23 \pm 0.081$. The corresponding value for the growth potency of 2 is 0.301 ± 0.098. The growth potency of extract boiled at pH 2.0 is 8.5 times its ketogenic potency. Logarithm $8.5 = 0.93$, and the standard error of the latter is $\sqrt{0.081^2+0.098^2}$, or 0.127. It may thus be predicted that 99 times in 100 the growth potency will be at least 4.01 times that of ketogenic potency. One may therefore conclude that the difference between the two potencies is highly significant.[1]

DISCUSSION

One criticism which might be raised against the reasoning offered above for the existence of two separate principles is that ketogenic activity may depend upon the simultaneous action of growth principle plus a more thermolabile synergist which is destroyed on heating. The magnitude of the decline in ketogenic potency after heating strongly suggests, however, that the substance which is destroyed is more than a supplementary synergist. It might also be objected that the loss of ketogenic activity in these experiments is due to the production through heating of an anti-ketogenic material which might be derived from a tissue extract. Certain experiments were performed which eliminated the likelihood of such a mechanism. An extract of kidney was prepared by the same method as that of Burn and Ling for pituitary extract. This preparation, when injected either unheated or after heating for 10 minutes at pH 2.0, did not affect the blood-acetone-body level in a group of 11 fasted rats. Even glucose, when injected in doses of 10 mg., produced only the small average decline of 0.9 mg. per cent in a series of 6 rats. The total protein contained in the highest dose of pituitary extract used in the assay of *B-2* did not exceed 4 mg.

Growth hormone has usually been considered a thermolabile substance, although very little evidence is available attesting to the ease with which it is destroyed by

[1] Dr. C. I. Bliss has very kindly calculated the above potencies and errors by more exact methods (6). The ratio of growth to ketogenic potency which he obtains is 10.5, and the chances are 99 in 100 that it will not be less than 4.27.

heat. Even though it is true that in the above experiments the hormone was almost completely destroyed by a short period of boiling in alkaline medium and was largely destroyed when in neutral solution, it was surprising to discover how much escaped destruction in a highly acid medium. It may be recalled, however, that Collip (9) from preliminary experiments suspected that the hormone would show maximum heat stability at pH 2.0. The deleterious effect of alkali has been previously noted by Evans, Meyer, and Simpson (10), who showed that growth activity is rapidly lost when extracts are incubated at 37 to 50° C. with the pH adjusted to 11 or 12.

Butts, Cutler, and Deuel (11) found that a pituitary extract (Burn and Ling) which had been boiled 5 minutes at pH 6.8 produced no ketonuria in a group of 4 fasting rats and concluded that the ketogenic principle was destroyed by heating. Neufeld and Collip (12), however, reported that ketogenic activity was retained in extracts which had been subjected to a boiling temperature in a medium containing N/10 HCl. The acidity of such a solution would probably approximate pH 2.0. Although the experiments of Neufeld and Collip indicated that ketogenic principle was not entirely lost after such treatment, the possibility of a substantial decrease in potency was not excluded. The doses of both heated and unheated extract which were employed were too high to detect partial inactivation. The dose of extract used by Neufeld and Collip was equivalent to 0.2 to 0.4 gm. of original gland, and this quantity exceeds by 1000 to 2000-fold the minimal dose of crude extract which will augment ketonemia in the fasting rat. A decrease in potency to 1/17 after heating would still leave a residual activity equivalent to 6 times the minimal stimulating dose. This activity is sufficient to stimulate a ketosis which, in degree, would not differ materially from that elicited by an unheated preparation, since the doses of both preparations lie in the region of maximal response.

There is now good agreement that ketogenic activity is rapidly lost during heating in alkaline medium (1, 12, 13).

It should be noted that the heat stability of a substance existing in a highly impure state may not be an accurate reflection of the behavior of the same substance in pure form. Hence, although the present curves serve well as evidence for the separate identity of growth and ketogenic principles, a demonstration of the final precise character of the pH-heat stability of these substances must await their chemical isolation.

SUMMARY AND CONCLUSIONS

The pH-heat-stability curves of growth and ketogenic activities of an anterior pituitary extract have been described. Although both activities show maximum resistance to destruction by boiling in acid solution, growth potency is significantly less affected than ketogenic potency at pH 2.0. This finding strongly suggests that growth and ketogenic effects are due to two separate principles.

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ULTRAFILTRATION OF ANTERIOR PITUITARY KETOGENIC AND GROWTH PRINCIPLES

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MAGISTRIS (1), Anselmino and Hoffman (2), and Denstedt, O'Donovan and Neufeld (3) have reported that the ketogenic principle of the anterior pituitary is ultrafiltrable. Shipley and Long (4) and Greaves, Freiberg, and Johns (5), however, could not demonstrate ketogenic activity in ultra-filtrates which had been obtained with collodion membranes. Evidence concerning pituitary growth hormone is also conflicting, for although this substance is thought to be a protein, Van Dyke and Wallen-Lawrence (6), employing a collodion barrier, reported one experiment in which activity was present in the dialyzate from an extract.

Ultrafiltrable substances are sometimes defined as those which will permeate membranes which retain proteins but admit substances of smaller molecular size. The term ultrafiltrable, however, has no such precise meaning for it is possible to prepare filters which retain not only molecules of protein but also those of smaller dimension, and, in turn, certain membranes will admit proteins of small molecular size but retain those composed of relatively large molecules. The average pore size of the membranes used in the experiments noted above was not given in the published reports, and no studies have been reported in which membranes of graded porosity have been employed in order to define the minimum pore size which allows the active molecules to pass. Evidence bearing upon the molecular size of the growth and ketogenic principles as obtained with graded ultrafilters is submitted in the present report.

METHODS

Graded cellophane filters were prepared by the method of Seymour (7). The poor adsorption capacity of cellophane for filtered material makes it superior to collodion for purposes of ultrafiltration. The range of pore size used extended from that which excluded all protein (1.6 mμ) to a size which passed molecules the size of those of hemoglobin and serum globulin (6.9 mμ). The pituitary extract employed was that described by Burn and Ling (8). Before use it was frozen and remelted in order to facilitate removal of the bulky gelatinous precipitate which invariably forms. The PH of the extract was 7.5.

The original extract and the ultrafiltrates were analyzed for total nitrogen and non-protein nitrogen. Protein nitrogen was determined by difference. Trichloracetic acid was used as the protein precipitant, and nitrogen was determined by Nesslerization. Ultrafiltration was carried out at room temperature and was completed within 4 to 9 hours. All preparations were preserved in the frozen state at −15° C.

The methods by which growth and ketogenic activity were determined, and the

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procedures for calculation of relative potency have been described in a previous publication (9). The groups of rats receiving untreated pituitary extract (Burn and Ling) in this previous work have been used as standard controls for the estimation of growth and ketogenic potencies of the filtrates described below. The same original extract was used in both experiments.

RESULTS

Ketogenic activity. Untreated cellophane, the finest filter used, possessed pores which averaged 1.6 mμ in diameter. It yielded a protein-free filtrate with a nitrogen

TABLE 1. KETOGENIC ACTIVITY

Extract	Total Nitrogen Content mg./cc.	NPN Content mg./cc.	Protein N Content mg./cc.	No. of Animals	Dose (μg. of total N)	Rise in Blood Acetone Bodies mg. %	Potency of Protein Fraction in Relation to that of Original Extract
Uninjected controls.				12		0.8±0.3[1]	
Original extract	1.39	0.28	1.11	14 37	10 20	3.3±0.8 5.7±0.5	
Pore size 1.6	0.25	0.25	0.00	10	500	1.3±0.7	
Pore size 3.3	0.30	0.28	0.02	10	330	7.1±0.9	1.05[2] (0.8 to 1.4)
Pore size 5.6	0.33	0.26	0.07	12	40	4.5±0.8	1.03 (0.8 to 1.3)
Pore size 6.9	0.39	0.28	0.11	13 4	40 80	2.4±0.7 5.1±1.5	0.42 (0.34 to 0.52)

[1] Standard error of the mean.

[2] Limits defined by standard error. It should be noted that this estimate of potency is also influenced by the error of the protein nitrogen determination, which, inasmuch as it is calculated by difference, may be rather large.

content of 0.25 mg. per cc. A dose of this preparation equivalent to 500 micrograms of nitrogen failed to produce any significant ketosis (table 1). The original extract produced a distinct response with a dose containing 20 μg. of total N, and a slight response with a 10-μg. dose. A filter with an average porosity of 3.3 mμ passed a small amount of protein (0.02 mg. of protein N per cc.). This filtrate was active. If one assumes that the activity of this preparation can be attributed exclusively to its protein component, a calculation of the potency of the protein material shows that it is neither more nor less active than the crude protein of the original extract. A filter of pore size 5.6 mμ passed a greater amount of protein but again there was no selective concentration of ketogenic principle in the filtered protein. The protein component obtained with a 6.9 mμ-pore filter was somewhat less potent than that obtained with the 5.6 and 3.3 mμ-pore filters.

Growth activity. The filtrate obtained with a 1.6 mμ-pore filter was devoid of growth activity (table 2). The only other filtered preparation which was available in sufficient quantity for growth assay was that from the 3.3 mμ-pore filter. Growth activity was present in this filtrate. There was a suggestion of selective filtration of growth principle, inasmuch as the protein fraction of the filtrate was 1.7 times as potent as that of the original extract.

DISCUSSION

Membranes of the type used in these experiments do not allow the passage of egg albumin when the pore size is 3.0 mμ, and if the size is increased to 4.2 mμ only a small portion of serum albumin is passed (7). In the present investigation, the 1.6 mμ-pore size passed neither protein nor active material, while the 3.2 mμ-pore filter admitted a trace of protein and the filtrate was definitely active both in producing growth and augmenting ketosis. It would, therefore, seem likely that the molecules of the growth and ketogenic principles are similar in size to those of egg albumin and serum albumin, and certainly are no larger than these.

Unfortunately, the proportion of inert protein which traverses the filter is very large, even when the pore size is adjusted to the lowest level which barely allows the

TABLE 2. GROWTH ACTIVITY

Extract	Total Nitrogen Content mg./cc.	Protein Nitrogen Content mg./cc.	Dose (μg. of total N)	Gain in Weight, gm. (6-day period)	Potency of Protein Fraction in Relation to that of Original Extract
Original extract	1.39	1.11	5	6.4±0.6 (14 animals)	
Pore size 1.6	0.25	0.00	250	−1 +1 −1 0 Av. −0.25	
			75	+3 +4 +8 +8 +12 Av. + 7.0	
Pore size 3.3	0.30	0.02	150	+12 +10 + 9 + 5 +17 Av. 10.6	1.7 (Limits defined by standard error = 1.3 to 2.3)

molecules of active hormone to pass. Porosities from 3.3 mμ up to a size which admits hemoglobin and serum globulin (6.9 mμ) do not yield preparations in which there is a selective concentration of ketogenic substance. Although a 3.3 mμ-pore filter yields a filtrate which, in terms of protein content, is relatively more potent in growth activity than crude extract, the increase is of small magnitude. Ultrafiltration, therefore, does not offer a satisfactory method for the purification and concentration of growth and ketogenic principles.

SUMMARY AND CONCLUSIONS

Ultrafiltration of anterior pituitary extract through cellophane filters of graded porosity yielded filtrates which possessed growth and ketogenic activity when the

pore size was just large enough to admit protein (3.3 mμ). When the pore size was small enough to exclude protein (1.6 mμ), there was no activity. It is therefore concluded that these principles, as they exist in the extract, are composed of molecules comparable in size to those of small molecular proteins. Ultrafiltration did not prove satisfactory as a method for the concentration of growth and ketogenic principles.

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FURTHER STUDIES OF THE VIDIAN GANGLION AS A SOURCE OF INNERVATION OF THE ANTERIOR LOBE OF THE HYPOPHYSIS

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BILATERAL removal of the sphenopalatine ganglion, in the rat, is followed by pseudopregnancy in 40 per cent of the cases (1). This result suggested a possible neural effect upon the gonadotropic function of the hypophysis. Investigation of the proximal nerve supply to the sphenopalatine ganglion disclosed (2) at the junction of the greater superficial petrosal nerve with the great deep petrosal nerve a small ganglion, the Vidian ganglion, from which small branches are distributed to the sixth nerve, to the internal carotid plexus, and to the sheath and probably the parenchyma of the anterior lobe of the hypophysis. The present study is concerned with the Vidian ganglion as a possible source of innervation to the anterior lobe, and with the physiological effects on the hypophysis of Vidian ganglionectomy.

Two aspects of hypophyseal function have been selected for study in animals in which the Vidian ganglia have been extirpated, *a*) the gonadotropic factor, with the production of pseudopregnancy as a criterion of disturbance of hypophyseal function, and *b*) the diabetogenic factor, with the alteration of insulin sensitivity the criterion. The production of pseudopregnancy (3) occurs only in the presence of functional corpora lutea, maintained in this instance by gonadotropic hormone from the anterior lobe. Change in sensitivity to insulin is a criterion of functional disturbance of the hypophysis because, from the work of many investigators (4–8), it is known that hypophysectomy gives rise to a marked increase in insulin sensitivity in starved animals. Dosages of insulin which have no effect on unoperated starved animals will cause hypophysectomized fasted animals to suffer hypoglycemic collapse and convulsions. The insulin sensitivity of rats in which the Vidian ganglia had been removed was investigated, to see whether a destruction of this possible source of innervation would in any way reduplicate the effects of hypophysectomy.

MATERIAL AND METHODS

Young adult female albino rats from an inbred colony were used. The animals ranged in weight from 160 to 215 gm.

For the pseudopregnancy experiments, all rats used had regular 4 or 5-day estrous cycles; no experimental or control procedures were begun until at least 4 consecutive regular cycles had been observed. Operations were performed on animals in all stages of the estrous cycle. Three types of operations were performed, *a*), bilateral removal of the Vidian ganglion and its sympathetic and parasympathetic roots; *b*), bilateral sectioning of the greater superficial petrosal nerve (parasympathetic root); and *c*), bilateral opening of the tympanic bulla, as a control operation, to test the effects of operative trauma alone.

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Bilateral removal of the Vidian ganglion. The extirpation of the Vidian ganglion requires the exposure and removal of the tympanic bulla, which lies lateral to the basi-sphenoid and occipital bones. Location of the bulla is accomplished by Thompson's (9) method for hypophysectomy. Two modifications, however, have been made. The first is the use of a tracheal cannula. After the initial midline incision in the skin of the neck is made, the subcutaneous tissue, salivary glands, and pretracheal muscles are freed and retracted to either side of the midline, to expose the trachea. A small incision (about 2 mm.) is made between approximately the fourth and fifth tracheal cartilages, and a small L-shaped glass cannula is inserted in the opening, with the base of the L directed caudally. This cannula allows the animal to breathe during later stages of the operation, when the trachea anterior to the cannula is retracted, and also permits any mucus to be sucked from the trachea during and at the close of the operation. After insertion of the cannula, the retracted pretracheal muscles are allowed to return to position, and help hold the cannula in place. Secondly, the anterior belly of the digastric muscle is not removed. Instead, the 2 bellies are separated by cutting the tendon of insertion, are turned back during the operation, and are reunited by suture at the conclusion of the procedure.

When the bulla is located, it is exposed, opened, and removed. By very careful chipping of the bone, the internal carotid artery is uncovered from the level of the spheno-occipital suture to the posterior lacerated foramen. The contents of the bulla and part of the internal carotid artery are now visible. The stapedius muscle is moved aside to expose the underlying greater superficial petrosal nerve. The nerve is followed ventrad to its junction with the great deep petrosal nerve, which lies on the internal carotid artery. The Vidian ganglion is found at the point of junction of the 2 petrosal nerves on the artery [[see figure 1 (1) and figures 1, 2, 3 (2)]], embedded in fibrocartilage which firmly fixes the ganglion to the vessel. The petrosal nerves are each severed, 3 or 4 mm. from the ganglion. The greater superficial petrosal is lifted and cut; the great deep petrosal is cut, and then stripped from the artery toward the ganglion. With gentle traction exerted on the 2 petrosals, the ganglion is separated from the artery and excised.

After the Vidian ganglion has been removed bilaterally, the two bellies of the digastric are reunited on either side by a suture through the cut tendon. The cannula is then removed and the tracheal incision is closed by a suture. Before closing the tracheal incision, it is advisable to apply suction within the trachea to remove any accumulation of mucus. If this is not done, the animals frequently die during subsequent closure, because of respiratory obstruction. The median neck incision is then closed, and suction is once more applied to the trachea through the mouth, to remove any remaining mucus.

The operation is performed under nembutal anesthesia. A Bausch and Lomb binocular dissecting microscope (×10) is used. The procedure is not a simple one. The operative field is small and deep, and requires brilliant, properly directed illumination. The major dangers lie in exposure of the internal carotid artery, and in freeing the Vidian ganglion from its very firm attachments to the artery. Tearing the internal carotid artery is followed by severe bleeding. Loss of blood from this artery, besides greatly endangering the animal's life, might also interfere with the hypophyseal blood supply and consequently give rise to spurious experimental results. Therefore, no animal which suffered serious bleeding was used for subsequent experiment.

Bilateral section of the greater superficial petrosal nerve. The operation is identical with the first operation through the step of opening the bulla. It is not necessary, however, to expose the internal carotid artery, for the greater superficial petrosal nerve may be found by pushing aside the stapedius muscle. The nerve is then lifted and a segment excised. The procedure for closing is as described in the first operation.

Bilateral opening of the tympanic bulla. The procedure described in the first operation for opening the bulla is followed.

In the insulin sensitivity experiments, a dosage of 3 units of insulin per kg. of body weight was used. This quantity was arrived at by testing a large group of normal, fasted rats with various dosages of insulin, ranging from 27 U per kg. to 3 U per kg.

With 3 U, injected subcutaneously, only 1 of a group of 30 rats had a convulsion. The rats were starved for 24 hours before injection of insulin (48-hour starvation periods were tried and found to be no more effective than 24-hour periods). The rats were observed for a period of 3 hours following injection. The onset of convulsions was characterized by lassitude and rapid breathing, later by unconsciousness and spasticity, and finally by the movements of actual convulsion. At the conclusion of the 3-hour post-injection period, even if animals showed severe symptoms of collapse and spasticity but no actual convulsions, the results were recorded as negative. Animals in convulsion were given 5 cc. of 20 per cent glucose by intraperitoneal injection; within 2 to 3 minutes of the glucose injection most of the rats were completely recovered, except for a mild lassitude. A small number of animals died after convulsion in spite of the glucose injection. Animals which did not have convulsions were nevertheless given 2 cc. of glucose in order to supply immediate nourishment after a long period of starvation.

RESULTS

Pseudopregnancy

A. Bilateral removal of the Vidian ganglion. Of a group of 22 animals, 17 were successfully operated. All 17 animals showed in the vaginal smear a prolonged leucocyte

TABLE 1. EFFECTS OF OPERATIVE PROCEDURES ON GONADOTROPIC FUNCTION OF THE HYPOPHYSIS

Operation	Number of Rats Operated	Average Weight, gm.	Postoperative Reaction			
			Estrous cycles		Deciduomata	
			Regular	Prolonged leucocyte phase	Uterus traumatized	Positive
Bilateral extirpation of Vidian ganglion	17	180	0	17	16	16
Bilateral section of greater superficial petrosal nerve	26	187	7	19	17	16
Bilateral removal of tympanic bulla	17	180	15	0	0	0

phase of the estrous cycle following operation. In all but 1 animal, leucocytosis started during the cycle in which the animal was operated; in 1 animal, there was 1 intervening cornified cell smear. Of the 17 successfully operated animals, 16 were used to test for decidual cell reaction. The uterus was traumatized on the fourth or fifth day of the prolonged leucocyte phase, by threading 1 horn longitudinally with a fine silk loop. Uterine biopsies were obtained from the animals on the fifth day after threading, and the biopsy specimens were sectioned for microscopic examination, as a check on the gross findings. Of the 16 animals threaded, all gave positive decidual reactions; 11 showed very large deciduomata which greatly thickened the uterus and almost obliterated the lumen; the remaining 5 rats showed smaller and more localized, but unmistakably positive, decidual growths (table 1).

B. Bilateral section of the greater superficial petrosal nerve. Twenty-six rats were operated. Of the 26 animals, 19 showed prolongation of the leucocyte phase of the vaginal cycle, following operation. Of the 19, leucocytosis appeared in the vaginal smear immediately after operation in 15 rats; in 2 animals there was 1 intervening smear with cornified cells, and in 2 others, leucocytosis appeared in the next cycle following operation. Of the 19 animals showing leucocytosis, 17 were threaded for detection of the decidual-cell reaction. Sixteen (94%) gave positive reactions; 15 deciduomata were very striking; 1 was smaller but clearly positive (table 1).

C. Bilateral opening of the tympanic bulla. In 15 rats in which the bullae were opened and partially removed, no animals showed any disturbance of the estrous cycle (table 1).

Insulin sensitivity

The various experiments performed were: *A*) Rats from which the Vidian ganglion and its roots had been removed bilaterally were starved for 24 hours and then given 3 U of insulin per kg. of body weight, subcutaneously. *B*) Rats from which a segment of the greater superficial petrosal nerve was removed bilaterally were starved for 24 hours and then given 3 U of insulin per kg. of body weight, subcutaneously. *C*) Rats in which the tympanic bulla was opened and removed bilaterally were starved for 24 hours and then given 3 U of insulin per kg. of body weight, subcutaneously (operative control). *D*) Unoperated rats which were starved for 24 hours were given 3 U of insulin per kg. of body weight, subcutaneously.

TABLE 2. INSULIN SENSITIVITY OF OPERATED RATS
(3 units of insulin per kg. of body weight. Food withdrawn 24 hours prior to the injections.)

Operation	Number of Tests	Number having Convulsions
Bilateral extirpation of Vidian ganglion	30	20 (66%)
Bilateral section of the greater superficial petrosal nerve	17	3 (17%)
Bilateral removal of tympanic bulla	17	3 (17%)
Unoperated controls	30	1 (3%)

Animals in *series A*, *B* and *C* received the initial insulin injection 1 or 2 days following operation; if they survived, the injections were repeated periodically during the extent of the experiments, some 2 or 3 times, some as often as 15 times, over a period of 7 months. Animals which had numerous injections did not, in general, have convulsions every time insulin was injected. In some animals, convulsions occurred early in the experiment, in others, not until later, and in still others, especially those which had very numerous injections, the occurrence of convulsions was more or less evenly distributed throughout the duration of the experiment. The results of all 4 series of experiments may be seen in table 2. In *experiment A*, in which the Vidian ganglion was removed bilaterally in 30 rats, 20 animals (66%) had convulsions at least once after insulin administration. The 1 convulsion which occurred in *experiment D* (unoperated starved rats) can most likely be attributed to an individual variation in sugar metabolism and storage. This may also partially explain the occasional convulsion seen in *experiments B* and *C* (section of the greater superficial petrosal nerve bilaterally and bilateral removal of the bulla); in addition, possible surgical injury may have been done, indirectly, to the Vidian ganglion.

DISCUSSION

The bilateral removal of the Vidian ganglion, in the rat, was followed by pseudopregnancy in all cases operated. The same procedure increased insulin sensitivity in

66 per cent of operated animals. It seems justified, therefore, in view of the anatomical studies (2), and the present data, to assume that the Vidian ganglion is a source of part of the innervation to the anterior lobe of the hypophysis, and that its removal, bilaterally, gives rise to alterations in hypophyseal function. Since pseudopregnancy is an indication of functional corpora lutea (which also can be produced by the administration of gonadotropic hormone), the genital effect following removal of the ganglia may be regarded as one of hypophyseal stimulation. On the other hand, the increased sensitivity to insulin postoperatively, paralleling as it does the effects of hypophysectomy, may be looked upon as hypophyseal inhibition. These results indicate, then, that, in the intact animals, both an inhibiting and stimulating effect reach the anterior lobe by means of the same pathway, that is, via the branches of the Vidian ganglion. The question arises whether both inhibition and stimulation are the result of a single impulse which the gland, by some power of selectivity (possibly depending on the type of cell reached), turns into one effect or the other, or whether the two different kinds of impulses reach the gland over pathways which are closely juxtaposed, as are for instance parasympathetic and sympathetic fibers in a peripheral nerve. As far as the time factor in the two types of experiment is concerned, both stimulating and inhibiting effects appear immediately postoperatively. The vaginal leucocytosis occurred, for the most part, in the same cycle in which the animal was operated, and the traumatization of the uterus was accomplished on the fifth day of leucocytosis. Because of this method of procedure, it is possible to say only that pseudopregnancy results as an immediate effect of Vidian ganglionectomy, no information being available from these experiments concerning a sustained stimulation of the gonadotropic factor from the anterior lobe. On the other hand, increased insulin sensitivity not only appeared immediately after operation, but was maintained for as long as 7 months after the ganglia were removed. As one part of the answer to the question, is the stimulation or inhibition really a single impulse, with the anterior lobe the final selector or are the two impulses separate, reaching the gland over a common nerve pathway, it at least may be said that either effect can occur immediately following Vidian ganglionectomy. A more satisfactory answer may be found in experiments now in progress.

In consideration of the alternate possibility that two different effects reach the gland over pathways that are closely juxtaposed, various workers (10, 11, 12) have demonstrated that nervous control of the gonadotropic function of the anterior lobe is not by way of the cervical sympathetics, by showing that rabbits in which the cervical sympathetic trunk has been sectioned bilaterally will, if allowed to mate, produce normal litters. While the nerve fibers of the infundibular stalk are regarded as the chief functional connection between the brain and the anterior lobe of the hypophysis (12, 13, 14), it has been demonstrated in this report that removal of the Vidian ganglia is followed by marked changes in hypophyseal gonadotropic function. It is seen (table 1) that both the removal of the Vidian ganglion and the section of the greater superficial petrosal nerve (parasympathetic root of the ganglion) are followed by a higher percentage of pseudopregnancies. On the other hand, it is shown (table 2) that although removal of the Vidian ganglion increases insulin sensitivity, sectioning of the greater superficial petrosal nerve does not. Additional experiments involving section of the great deep petrosal nerve in both the pseudopregnancy and the insulin sensitivity studies are planned.

SUMMARY

Previous anatomical studies in the rat (2) have suggested that the Vidian ganglion, found at the junction of the greater superficial petrosal and great deep petrosal

nerves, is a source of part of the innervation of the anterior lobe of the hypophysis. The physiological effects on the hypophysis of Vidian ganglionectomy in the rat have been investigated. Two aspects of hypophyseal function were selected for study: the gonadotropic factor, with the production of pseudopregnancy as a criterion of disturbance of hypophyseal function, and the diabetogenic factor, with the alteration of insulin sensitivity the criterion. The bilateral removal of the Vidian ganglion was followed by pseudopregnancy in all cases operated (table 1). The same procedure increased insulin sensitivity in 66 per cent of operated animals (table 2). It is concluded, in view of the anatomical studies (2) and the present data, that the Vidian ganglion is a source of part of the innervation to the anterior lobe of the hypophysis, and that its removal, bilaterally, gives rise to alterations of hypophyseal function. In addition, it is seen that sectioning of the greater superficial petrosal nerve (parasympathetic root of the ganglion) gives rise to a high percentage of pseudopregnancies (table 1), but does not alter insulin sensitivity (table 2). The implications of these various results are discussed.

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ACTION OF GYNECOGENIC AND ANDROGENIC HORMONES ON THE GONADS AND PITUITARY GLAND OF MATURE MALE RATS

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THE ACTION of the female sex hormone on male gonads has already been studied in detail in animal experiments (1–7). The results of the work reported to date may be summarized as follows. Prolonged treatment of the male with the female sex hormone produces in all cases an inhibition of the gonadotropic function of the anterior lobe of the pituitary with subsequent atrophy of the sex organs. The female sex hormone, thus, has an anti-masculine effect, but the involutional manifestations are limited in duration and disappear after discontinuation of the treatment. The pituitary gland of male animals so treated shows an increase in the number of the chromophobe cells, degranulation of the chromophils, and a decrease in the eosinophils with congestion of the capillaries.

Whereas the experiments of the above workers gave consistent results, investigations which were undertaken in an attempt to inhibit the anti-masculine effect of the female hormone, or to accelerate regeneration after discontinuation of treatment with estrogen, often gave very contradictory results. The reason for this probably lies in the fact that the investigators did not use animals of the same age, did not give equal doses of hormone, nor start their investigations at the same time; such results thus offer no possibility of comparison.

In this paper we have studied the action of the male sex hormone on the gonads and pituitary gland of mature male rats after preliminary treatment with female hormone.

MATERIAL AND METHOD

Mature albino rats of the Glaxo-Roche strain, most of which were bred in our own laboratories, were chosen for the investigations. The animals were divided into experimental groups of 8, 9, or 10 rats each, for each dosage level used. A total of about 250 rats was used. The animals in each group were litter-mates or, at the most, taken from two litters, and were kept in separate cages under the same conditions of light and temperature. They were fed alternately on fresh vegetables, meat, bread and milk or a flour and salt mixture (8) the composition of which guaranteed a sufficient intake of vitamins.

Special attention must be paid to the care and feeding of animals used in such experiments, in order to eliminate the possibility of injurious influences on the testes.

Untreated animals of the same litter served as controls and unilateral castrations were performed before and sometimes during treatment.

The female sex hormone used was estradiol dipropionate;[1] the male sex hormone was testosterone propionate.[1] These were administered subcutaneously.

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[1] The estradiol dipropionate (Di-Ovocylin) and testosterone propionate (Perandren) were supplied by the Society of Chemical Industry in Basle (Ciba).

In the first series of experiments the animals received 20 mg. of estradiol dipropionate within 20 days; in the second series, 20 mg. of estradiol dipropionate and 20 mg. of testosterone propionate; in the third, 20 mg. of estradiol dipropionate and 30 mg. of testosterone propionate; in the fourth series, 20 mg. of estradiol dipropionate and 40 mg. of testosterone propionate; in the fifth series, 30 mg. of testosterone propionate only. All of the dosages employed in the second, third and fourth series of experiments were given within a period of 30 days. In the fifth series, treatment lasted 10 days. In addition, one testis was removed in two series of experiments, after treatment with 20 mg. of estradiol dipropionate over a period of 20 days; following this 30 or 40 mg. of testosterone propionate was administered for 10 days, at the end of which time the second testis was removed.

The testes were prepared for histologic study by fixation in a mixture of corrosive sublimate-formalin-glacial acetic acid and embedded in methyl benzoate-celloidin or benzene and paraffin; blocks of tissue were cut at 4 to 5 μ and stained with hematoxylin and eosin, iron hematoxylin and azan. The pituitary glands were fixed in Helly's or Bouin's fluid and embedded in methyl benzoate-celloidin or benzene and paraffin; sections were cut at 2.5 to 3.5 μ and stained with hemalum and eosin; cloth fast yellow (Wallart) and Kresazan (Romeis) were also used (9).

When animals are killed with coal-gas, spontaneous ejaculation occurs which we have designated as the 'agony test'; the ejaculate was always tested for the presence of spermatozoa.

RESULTS

Spermiogenesis in the rat may be described in 4 distinct phases: *a*) proliferation; indirect division of the spermatogonia; *b*), growth; development of the spermatocytes from spermatogonia and an increase in size of the former up to the point of division; *c*), maturation, equatorial division; reduction division; *d*), transformation of the spermatids into spermatozoa in three stages.

Course of the degeneration and regeneration of the testis due to hormonal action. In judging histologically the degree of degeneration we utilized Schinz and Slotopolsky's classification of testicular atrophy (10). In all testicular injuries (mechanical, thermic, operative), testicular atrophy begins with a cessation of the transformation of spermatids into spermatozoa and this stage is designated as atrophy of the first degree.

Three degrees of atrophy are recognized. 1). No spermatozoa are seen in any of the tubules. The spermatids disappear with the formation of giant cells. The earlier stages of development of spermiogenesis remain. 2). No spermatozoa, spermatids or prespermatids are seen in any of the tubules. Only spermiocytes which do not divide are present. 3). A distinction is made between an early and a late stage of this third stage of atrophy. Common to both is that constrictions occur in seminiferous tubules simultaneously with their shrinkage as a result of a thickening and hyaline swelling of the membrana propria. In the early condition we find, in addition to spermatogonia at rest, some in a state of division, but their transformation into spermatocytes does not take place. In the late condition no spermatogonia are dividing; here and there are isolated spermatogonia undergoing necrobiosis. In all three stages of atrophy, basal cells are present.

Atrophies of fourth and fifth degree, observed by Schinz and Slotopolsky (10), which lead to complete destruction of testicular tissue after treatment for a number of years, have not been observed in our investigations.

Regeneration begins with the re-appearance of spermatocytes from spermatogonia, further development of spermatids, and finally of the spermatozoa.

The results are given in tabular form. The initial and final weights of the animals,

TABLE 1. DATA ON CONTROL, NORMAL MATURE MALE RATS

Initial Body Weight	Final Body Weight	Weight of Testes		Weight of Epididymides		Histological Condition			Agony Test
		T_1	T_2	E_1	E_2	Testis	Epididymis	Pituitary	
gm.	*gm.*	*mg.*	*mg.*	*mg.*	*mg.*				
176	222	1450	1500	500	500	Normal	Full of spermatozoa	Normal	Positive
187	200	1130	1350	560	530	Normal	Full of spermatozoa	Normal	Positive
162	180	1350	1350	450	450	Normal	Full of spermatozoa	Normal	Positive
204	254	1560	1650	530	500	Normal	Full of spermatozoa	Normal	Positive
190	235	1490	1470	570	540	Normal	Full of spermatozoa	Normal	Positive
187	240	1495	1480	505	520	Normal	Full of spermatozoa	Normal	Positive
180	210	1300	1320	430	450	Normal	Full of spermatozoa	Normal	Positive
160	192	1250	1230	410	420	Normal	Full of spermatozoa	Normal	Positive
175	208	1200	1280	310	350	Normal	Full of spermatozoa	Normal	Positive
185	215	1270	1120	380	350	Normal	Full of spermatozoa	Normal	Positive

weights of the testes and epididymides, their histological condition and that of the pituitary gland and the result of the 'agony test' are recorded.

Control animals (table 1). All animals increased in weight during the 30 days of the investigation. The weight of the testicles varied between 1330 and 1560 mg., those of the epididymis between 310 and 570 mg. Histologically, the seminiferous tubules showed all stages of spermatogenesis and the epididymis a variable degree of filling with spermatozoa. The pituitaries were normal in appearance. The agony test was positive in all animals, that is, spontaneous ejaculation occurred when they were killed with coal-gas.

Estradiol dipropionate (table 2). All animals decreased in weight after treatment with 20 mg. of estradiol dipropionate. The weights of the testes decreased considerably to about one-seventh to one-sixth of their normal weight. The epididymides were about one-fifth to one-fourth of their original weight. Histologically, there was atrophy of the second or third degree in the seminal epithelium; the epididymides were empty; the pituitaries, hyperemic; there was an increase in the number of chromophobe cells and hypertrophy of the same, with a decrease in the chromophils. The agony test was negative in all cases.

Estradiol dipropionate followed by 20 mg. of testosterone propionate (table 3). The body-weight of the rats had also decreased with this type of treatment. The testes, on the other hand, showed a less pronounced decrease in weight than with estradiol dipropionate alone, about one-fourth to one-third of the control values; the epididymides about one-third to one-half.

TABLE 2. EFFECT OF 20 MG. OF ESTRADIOL DIPROPIONATE GIVEN WITHIN 20 DAYS ON THE TOTAL WEIGHT OF TESTES, EPIDIDYMIDES AND PITUITARY GLANDS OF MATURE RATS

Initial Body Weight	Final Body Weight	Weight of Testes		Weight of Epididymides		Histological Condition			Agony Test
		T_1	T_2	E_1	E_2	Testes	Epididymis	Pituitary	
gm.	*gm.*	*mg.*	*mg.*	*mg.*	*mg.*				
173	140	250	300	81	100		Empty	Increase in	Negative
182	154	260	290	90	90		Empty	the chromo-	Negative
158	126	250	280	100	100		Empty	phobe cells;	Negative
175	132	220	230	100	100	Atrophy	Empty	hypertrophy	Negative
157	120	200	210	100	100	2nd–	Empty	of same with	Negative
154	144	220	230	80	80	3rd	Empty	decrease in	Negative
186	162	280	270	120	120	degree	Empty	the chromo-	Negative
156	124	250	240	100	95		Empty	phils;	Negative
176	160	270	270	100	90		Empty	hyper-	Negative
164	110	90	70	80	80		Empty	emia	Negative

TABLE 3. ACTION OF 20 MG. OF ESTRADIOL DIPROPIONATE, ADMINISTERED FROM THE 1ST TO THE 20TH DAY, AND OF 20 MG. OF TESTOSTERONE PROPIONATE, ADMINISTERED FROM THE 20TH TO THE 30TH DAY, ON THE BODY WEIGHT AND TESTES, EPIDIDYMIDES AND PITUITARY OF MATURE RATS

Initial Body Weight	Final Body Weight	Weight of Testes		Weight of Epididymides		Histological Condition			Agony Test
		T_1	T_2	E_1	E_2	Testis	Epididymis	Pituitary	
gm.	gm.	mg.	mg.	mg.	mg.				
160	115	415	420	190	195		Almost empty	Hypertrophy	Negative
180	178	550	550	210	205		Almost empty	of the	Negative
170	143	460	450	180	180	Atrophy	Almost empty	chromo-	Cells+
158	155	530	520	220	200	of first	Almost empty	phobe	Negative
165	147	450	450	160	150	degree	Almost empty	cells;	Negative
158	190	280	300	100	100	to normal	Almost empty	decrease	Negative
167	155	430	420	160	155		Almost empty	in eosino-	Negative
152	134	420	430	190	180		Almost empty	phils	Negative

Histological examination revealed after 30 days only atrophy of the first degree in the testes. In the epididymides there were only isolated spermatozoa with scanty cellular constituents. The pituitaries showed a proliferation and hypertrophy of the chromophobes with an increase in the eosinophils.

Estradiol dipropionate followed by 30 mg. of testosterone propionate (table 4). The rats showed a decrease in body weight which, however, was less than in the previous experiments; in one animal there was an increase in weight. The weights of the testes and epididymides showed no essential deviation from the values in table 3.

Histological examination revealed atrophy of the first degree only in the tubules. Most of the epididymides were empty, some contained scanty, and some numerous cellular constituents (prespermatids, spermatids and giant cells). The pituitary glands showed predominantly hypertrophic chromophobe cells and no decrease in the eosinophils. The agony test was negative; isolated cases showed cells and detritus in the smear preparations.

Estradiol dipropionate and 40 mg. of testosterone propionate (table 5). All of the animals, with one exception, showed a decrease in weight. The testicular weights had the same values as in the animals reported in table 4; the weights of the epididymides were reduced to about half of the control value.

Histological examination revealed, apart from atrophy of the first degree, normal conditions in the tubules of the testes. The epididymides contained spermatozoa in addition to cellular constituents. There was some hypertrophy of the chromophobe cells of the pituitary. The cytoplasm was paler; the chromophils showed no change.

TABLE 4. ACTION OF 20 MG. OF ESTRADIOL DIPROPIONATE, ADMINISTERED FROM THE 1ST TO THE 20TH DAY, AND OF 30 MG. OF TESTOSTERONE PROPIONATE, ADMINISTERED FROM THE 20TH TO THE 30TH DAY, ON THE BODY WEIGHT, TESTES, EPIDIDYMIDES AND PITUITARY OF MATURE RATS

Initial Body Weight	Final Body Weight	Weight of Testes		Weight of Epididymides		Histological Condition			Agony Test
		T_1	T_2	E_1	E_2	Testis	Epididymis	Pituitary	
gm.	gm.	mg.	mg.	mg.	mg.				
170	155	460	460	160	150		Cells sparse	Proliferation	Negative
168	171	380	410	170	160		Cells sparse	and hyper-	Cells+
165	134	370	370	150	140	Atrophy	Cells sparse	trophy of	Cells+
169	160	350	350	150	160	of the	Cells sparse	chromophobe	Cells+
162	147	450	450	170	150	first	Adhering cells	cells;	Cells+
160	162	470	470	220	220	degree	Adhering cells	no decrease	Cells+
159	139	420	430	170	160		Empty	in the eosino-	Negative
148	125	390	380	170	170		Empty	phils	Negative

TABLE 5. ACTION OF 20 MG. OF ESTRADIOL DIPROPIONATE, ADMINISTERED FROM THE 1ST TO THE 20TH DAY, AND OF 40 MG. OF TESTOSTERONE PROPIONATE, ADMINISTERED FROM THE 20TH TO THE 30TH DAY, ON THE BODY WEIGHT, TESTES, EPIDIDYMIDES AND PITUITARY OF MATURE RATS

Initial Body Weight	Final Body Weight	Weight of Testes		Weight of Epididymides		Histological Condition			Agony Test
		T_1	T_2	E_1	E_2	Testis	Epididymis	Pituitary	
gm.	gm.	mg.	mg.	mg.	mg				
160	164	450	430	270	280		Cells	Proliferation	Negative
160	136	480	470	250	245		Cells	and hyper-	Negative
158	140	510	500	270	270	Atrophy	Spermatozoa	trophy of	Positive
156	135	450	350	240	230	of the	Spermatozoa	chromophobe	Positive
152	132	400	410	270	270	first	Spermatozoa	cells; cyto-	Positive
155	150	420	415	250	245	stage	Spermatozoa	plasm paler;	Positive
170	160	500	510	270	265	to normal	Spermatozoa	chromo-	Positive
175	155	490	480	240	240		Spermatozoa	phils	Positive
168	140	450	435	270	275		Spermatozoa	unchanged	Positive

Testosterone propionate (table 6). In this series all of the rats increased in body weight. The testes showed a slight decrease in weight, whereas the weights for the epididymides were normal.

Histological examination revealed almost normal conditions in the seminal epithelia; the epididymides were full of spermatozoa. The pituitary glands were normal. The agony test was positive in all cases.

Unilateral castration experiments (tables 7 and 8). The body weight of the animals showed a decrease after the experiment recorded in table 7. The weights of the first testis and epididymis showed approximately the same decrease as in the experiment with estradiol dipropionate alone. On the other hand, the second testis, after the animal was treated further with 30 mg. of testosterone propionate following unilateral castration, showed almost uniformly an increase in weight to double its original value; the increase in weight was less in the case of the second epididymis.

Histological examination showed atrophy of the 2nd and 3rd degree occurring in the first testis after treatment with estradiol dipropionate. The first epididymis was practically empty. The second testis, on the other hand, was almost normal, with occasionally an instance of atrophy of the first degree. The second epididymis was sometimes empty, sometimes filled with cellular constituents. The pituitaries showed, in addition to proliferation and hypertrophy of the chromophobes, no decrease in the eosinophils. The agony tests were negative.

There was a decrease in the body weight of the rats used in the experiment recorded in table 8. The weights of the first testis and epididymis were approximately

TABLE 6. ACTION OF 30 MG. OF TESTOSTERONE PROPIONATE, ADMINISTERED FROM THE 1ST TO THE 10TH DAY ON THE BODY WEIGHT, TESTES, EPIDIDYMIDES AND PITUITARY OF MATURE RATS

Initial Body Weight	Final Body Weight	Weight of Testes		Weight of Epididymides		Histological Condition			Agony Test
		T_1	T_2	E_1	E_2	Testis	Epididymis	Pituitary	
gm.	gm.	mg.	mg.	mg.	mg.				
147	167	950	980	340	345		Full of spermatozoa	Normal	Positive
159	190	1150	1140	460	450		Full of spermatozoa	Normal	Positive
143	157	1050	1050	425	410		Full of spermatozoa	Normal	Positive
146	165	1100	1130	430	460	Almost	Full of spermatozoa	Normal	Positive
150	170	1050	1020	400	410	normal	Full of spermatozoa	Normal	Positive
147	165	1150	1150	480	375		Full of spermatozoa	Normal	Positive
140	162	1000	1000	410	415		Full of spermatozoa	Normal	Positive
150	170	1110	1115	440	435		Full of spermatozoa	Normal	Positive

TABLE 7. EFFECT OF 20 MG. OF ESTRADIOL DIPROPIONATE, ADMINISTERED FROM THE 1ST TO THE 20TH DAY, ON THE FIRST TESTIS AND EPIDIDYMIS. AFTER UNILATERAL CASTRATION, EFFECT OF 30 MG. OF TESTOSTERONE PROPIONATE FROM THE 22ND TO THE 31ST DAY ON THE SECOND TESTIS, EPIDIDYMIS, PITUITARY AND ON THE BODY WEIGHT OF MATURE RATS

Initial Body Weight	Final Body Weight	Weight of Testes		Weight of Epididymides		Histological Condition					Agony Test
		T_2	T_1	E_2	E_1	Testis T_1	Epididymis E_1	Testis T_2	Epididymis E_2	Pituitary	
gm.	gm.	mg.	mg.	mg.	mg.						
168	155	390	520	90	120	Atrophy 2	Empty	Almost normal	Cells	Proliferation and hypertrophy of chromophobe cells; no decrease in eosinophils	Negative
150	140	310	610	120	150	Atrophy 2	Empty	Almost normal	Cells		Cells+
172	160	270	600	100	150	Atrophy 2	Empty	Atrophy 1-normal	Almost empty		Negative
150	120	300	600	110	150	Atrophy 2–3	Cells	Atrophy 1	Cells		Negative
166	150	315	600	120	150	Atrophy 2–3	Empty	Atrophy 1	Empty		Negative
172	160	280	590	130	160	Atrophy 2	Empty	Atrophy 1-normal	Empty		Negative
168	153	300	610	110	140	Atrophy 2	Empty	Atrophy 1-normal	Empty		Negative
187	170	340	630	100	150	Atrophy 2	Empty	Atrophy 1-normal	Empty		Negative
188	154	290	615	115	180	Atrophy 2	Cells	Almost normal	Cells		Negative

the same as those recorded in table 7. The weight of the second testis of the animals treated with 40 mg. of testosterone propionate, however, was greater than in the case of those treated with 30 mg. The weights of the epididymis were also somewhat higher.

Histological examination showed in the first testis an atrophy of grades 2 to 3. The epididymides were empty. The second testis was normal in one-half of the animals, in the others there was atrophy of the first degree or the tubules were normal. The

TABLE 8. EFFECT OF 20 MG. OF ESTRADIOL DIPROPIONATE, ADMINISTERED FROM THE 1ST TO THE 20TH DAY, ON THE FIRST TESTIS AND EPIDIDYMIS. AFTER UNILATERAL CASTRATION, EFFECT OF 40 MG. OF TESTOSTERONE PROPIONATE, ADMINISTERED FROM THE 22ND TO THE 31ST DAY, ON THE TESTES, EPIDIDYMIDES, PITUITARY AND BODY WEIGHT OF MATURE RATS

Initial Body Weight	Final Body Weight	Weight of Testes		Weight of Epididymides		Histological Condition					Agony Test
		T_1	T_2	E_1	E_2	Testis T_1	Epididymis E_1	Testis T_2	Epididymis E_2	Pituitary	
gm.	gm.	mg.	mg.	mg.	mg.						
150	130	300	510	100	130	Atrophy2	Empty	Atrophy 1 to normal	Cells	Hypertrophy of chromophobes with palecytoplasm; no change in the chromophils	Negative
150	135	320	500	100	120	Atrophy 2	Empty	Normal	Spermatozoa		Positive
186	175	250	750	100	120	Atrophy 2–3	Empty	Normal	Spermatozoa		Positive
160	136	250	500	90	110	Atrophy 2–3	Empty	Atrophy 1	Cells		Negative
154	140	280	615	120	135	Atrophy 2–3	Empty	Normal	Spermatozoa		Positive
165	147	270	600	140	100	Atrophy 2–3	Empty	Normal	Spermatozoa		Positive
150	135	310	615	90	130	Atrophy 2–3	Empty	Normal	Spermatozoa		Positive
152	145	295	580	110	120	Atrophy 2–3	Empty	Normal	Spermatozoa		Positive

pituitary glands showed hypertrophied chromophobes with pale cytoplasm, without changes in the chromophils. The agony test was positive in most instances.

DISCUSSION

Response of body weight and the weights of the testis and epididymis of the experimental animals. In contrast to the control animals, all of which increased in weight within the 30-day period of the investigation, all animals treated with estradiol dipropionate alone or in combination with testosterone propionate show a decrease in weight. Only those animals treated with 30 mg. of testosterone propionate alone showed an increase in weight. It is apparent that testosterone propionate, after preliminary treatment of mature male rats with estradiol dipropionate, has neither a compensating nor a promoting effect on the body weight of the rat.

With reference to the weights of the testes we find, in comparison with the control animals, the most pronounced decrease in weight (one-seventh to one-sixth of

the normal weight) in rats which received only estradiol dipropionate (see reference 10). The decrease in weight in the animals treated with estradiol dipropionate and testosterone propionate is proportional to the dosage of the latter and is one-fourth to one-third of the mean weight of the controls. The rats treated with testosterone propionate alone show a small decrease in testicular weight (12). In the last two series of experiments (tables 7 and 8), the testes show the same decrease in weight after treatment with estradiol dipropionate as in the previous experiments. In the remaining testis after unilateral castration the increase in weight after treatment with testosterone propionate is equal to about one-half the normal weight.

Testosterone propionate thus seems to have a stimulating effect on the testicular

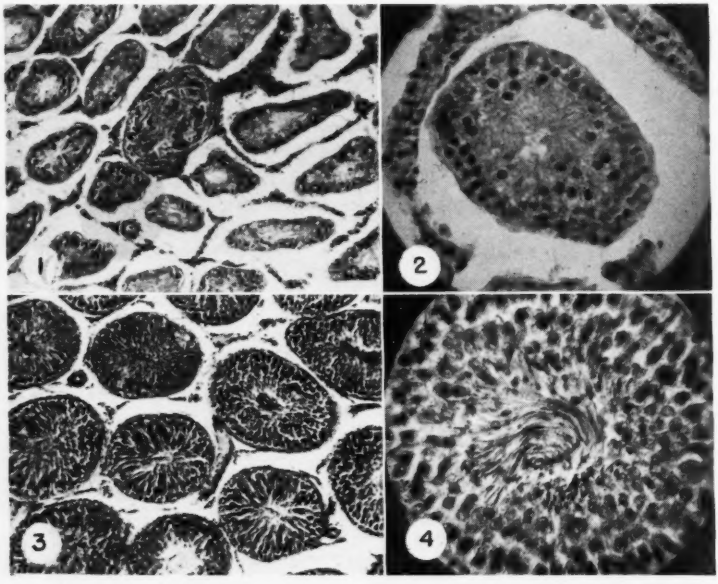

Fig. 1. Effect of 20 mg. of estradiol dipropionate administered from the first to the 20th day, on mature rat testes. Atrophy of grade 2 and 3. Fig. 2. Enlargement of one of the tubules shown in figure 1.

Fig. 3. Effect of 20 mg. of estradiol dipropionate administered from the first to the 20th day, 40 mg. of testosterone propionate administered from the 21st to the 30th day, on mature rat testes. Normal tubules. Fig. 4. Enlargement of the tubules shown in figure 3.

weight after previous treatment with estradiol dipropionate. This becomes particularly evident in the unilaterally castrated animals. On the other hand, the administration of testosterone propionate alone is accompanied by a slight decrease in weight which can be explained by the fact that the male hormone acts only on the injured testis, whereas the normal testis undergoes no particular change. It is, however, reasonable to consider the possibility that the normal testicular weights of rats which have been pre-treated with estradiol dipropionate may be regained with testosterone propionate by prolonging the duration of treatment.

The greatest decreases in the weights of the epididymides in comparison with the control animals (one-fifth to one-fourth of the normal values), are again obtained in rats treated with estradiol dipropionate. After combined treatment with estradiol

dipropionate and testosterone propionate, the epididymis has a weight of one-third to one-fourth of the normal value. In the animals treated with testosterone propionate, the weights of the epididymis are almost normal.

In the unilaterally castrated animals the increase in weight of the epididymis after treatment with testosterone propionate is less marked than in the case of the testis. In general, however, the results can be compared with those obtained on the testes; the deviations may be explained by the variation in the degree of filling of the epididymis.

Histological changes. Even though the weights of the animals and their gonads allow a certain insight into the action of the hormones administered, they are not so characteristic as the histological changes.

Confirming the work of other workers, we find atrophy of the 2nd and 3rd degree in the testis after treatment with estradiol dipropionate (fig. 1, 2); the epididymis is empty. The condition of the testes of the animals treated with estradiol dipropionate

SUMMARY OF TABLES 1 TO 6

Table Number	Dosage, mg.	Mean Value		Percentage Change in Body Weight	Mean Testicular Weight, mg.	Percentage Decrease in Testicular Weight
		Initial body weight, gm.	Final body weight, gm.			
1	controls	180.6	215.6	+19.4	1362.0	
2	20, estradiol dipropionate	168.1	137.2	−17.2	234.0	82.8
3	20, estradiol dipropionate +20, test. prop.	163.7	152.1	− 7.1	442.2	67.5
4	20, estradiol dipropionate +30, test. prop.	162.6	149.1	− 8.3	413.1	69.7
5	20, estradiol dipropionate +40, test. prop.	161.5	145.8	− 9.7	452.7	66.8
6	30, test. propionate	147.7	168.2	+13.9	1071.5	21.3

and testosterone propionate varies from normal to atrophy of the first degree in the tubules (fig. 3, 4). Some of the epididymides are empty, others are filled with cellular constituents. Testes and epididymides of rats which received only testosterone propionate are almost normal. The results in the unilaterally castrated animals are the same as those obtained in the epididymides and pituitaries of the animals which had received combined treatment.

The results of the histological examination of the pituitary confirm the results of other workers in that we found, after treatment with the female sex hormone, an increase in the number of chromophobes and hypertrophy of same, with decrease in the chromophils, together with general hyperemia.

After treatment with female and male sex hormones, the effects of the female hormone can be counteracted to an extent depending on the dose of male hormone (20, 30 or 40 mg. of testosterone propionate), with the exception of the hypertrophied chromophobes in the pituitary which are still present after 40 mg. of testosterone propionate.

The administration of male sex hormone alone did not affect the pituitary.

The unilateral castration carried out in two series of experiments after treatment with female sex hormone had no particular effect on the condition of the pituitary.

Sperm test. The results of the agony tests agree with the histologic condition of the epididymis. In all cases in which the epididymis was empty histologically, the

agony test was also negative. This was true in all animals after treatment with estradiol dipropionate. After combined treatment with estradiol dipropionate and testosterone propionate, however, there were cellular constituents in the smear preparations and sometimes spermatozoa, as in the epididymis. In the animals treated with testosterone propionate alone, the agony test was positive, as it was the normal controls.

SUMMARY

It is known from previous investigations (13) that rat testes undergo atrophy of the second or third degree after treatment with high dosages of female hormone. The testes may recover spontaneously, for which 20 to 24 weeks are required.

From the above results is seen the unequivocal action of the male hormone in accelerating regeneration after treatment with female hormone; this action becomes apparent, within 10 days, on the testis, epididymis and pituitary gland of mature rats. The male hormone has, however, no visible effect on the histological picture of the gonads and pituitary in normal animals.

In general we have succeeded, in the experimental arrangement which we have chosen, in counteracting the changes caused by the female hormone by administering male hormone; it must be emphasized that whereas we succeeded in producing entirely normal conditions again in the testis, the pituitary gland always showed some effect of the female hormone action in the form of hypertrophied chromophobes.

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AN ANDROGENIC SUBSTANCE IN FECES FROM CATTLE AS DEMONSTRATED BY TESTS ON THE CHICK

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THE CHIEF NATURAL SOURCES of androgenic substances have been male and female human urine and testes (1, 2). After comparing the male-hormone content of urine from several sources, Koch (1) reported relatively high concentrations in male human urine (40–100 I.U. per 24 hours) and female human urine (30–100 I.U. per 24 hours) as compared with 8, less than 1, and 4 I.U. per liter in urines from the stallion, bull, and ram, respectively. Although, according to Dohrn and Faure (3), considerable quantities of estrogens are excreted in the feces of gravid women, there appears to be no information concerning the excretion of androgens in the feces.

In the course of experiments on chick nutrition (4) at the Beltsville Research Center to determine whether or not feces of cattle could be used as a source of certain members of the vitamin B-G complex, one of us (J. C. H.) observed precocious comb and wattle growth in chicks when their diet was supplemented with either dried feces from cows or a 60 per cent alcohol extract of this material. As a result of this observation a series of three experiments was undertaken to ascertain *a*) the presence or absence of the active factor in the feces of cattle of different sexes and ages, *b*) its source, and *c*) its nature.

MATERIALS AND METHODS

One-day-old Rhode Island Red chicks, brooded in electrically heated brooders, were used as the experimental animals. Six different lots of 25 chicks each were used in the first experiment, 8 lots of 25 each in the second, and 6 lots of 25 each in the third.

Two basal diets were fed to the chicks. One, designated *basal diet* A, was comparatively simple and of low riboflavin content; the other, designated as *basal diet B*, was a well-balanced, all-mash starting-and-growing diet. *Basal diet* A was used in all three experiments, *diet B* was fed to only two pens in the first experiment and was not used in either the second or third experiment.

Urine-free feces from cattle were collected from four different sources: *a*), lactating cows, on pasture, in various stages of gestation; *b*), lactating cows, without access to pasture, in various stages of gestation; *c*), unbred heifers, without access to pasture; and *d*), mature bulls having limited access to pasture. All of the fecal material, with one exception, was dried in thin layers in an electrically-heated drying oven at about 45 to 47° C. for 24 hours and then ground to a meal before being added to the basal diets. The exception was the feces from the cows without access to pasture which were dried in comparatively thick layers at about 80° C. for 48 hours.

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The several types of dried feces, the extracts made from some of them, the resulting residues, and the other preparations that were used in these experiments were carefully mixed with the diets before they were fed to the chicks. The quantities used and other pertinent information are given in the following description of the experiments and in the tables.

EXPERIMENTAL WORK AND RESULTS

Experiment 1. In this experiment the effect of the addition of 10 per cent of feces from cows (with access to pasture), heifers, and bulls, respectively, to the diet of the chicks was observed. The pertinent observations are given in the first section

TABLE 1. SUPPLEMENTS TO THE DIET, AVERAGE LIVE WEIGHTS OF CHICKS AT 4 WEEKS OF AGE, AND AVERAGE WEIGHTS OF THE COMBS, TESTES, AND OVARIES

Lot No.	Basal Diet	Additions to Basal Diet, %	Number		Average Live Weight, gm.		Average Weight of Combs, mg.				Average Weight of Gonads, mg.	
							Males		Females			
			Males	Females	Males	Females	Wt., mg.	% Increase	Wt., mg.	% Increase	Testes	Ovary
		Chicks used in experiment 1										
5	A	None (negative control)	8	9	147.3	163.3	60.7		37.7		34.7	51.9
6	A	Cow feces (access to pasture) 10	13	11	162.3	157.9	267.5	341	176.5	368	21.2	34.1
7	A	Heifer feces 10	13	11	175.1	141.8	130.0	114	60.6	61	27.7	43.3
8	A	Bull feces 10	8	15	156.8	136.5	60.2	− 1	31.5	−16	31.4	37.8
9	B	Cow feces (access to pasture) 10	10	13	232.7	225.8	250.3	97	209.6	375	30.6	50.9
10	B	None	10	15	247.6	220.2	127.3		44.1		51.6	60.3
		Chicks used in experiment 2										
11	A	Cow feces (access to pasture) 10	14	10	178.0	164.7	286.8	345	219.8	509	22.5	37.2
12	A	Cow feces (without pasture) 10	10	14	140.5	152.6	54.3	−16	39.5	9	28.2	46.6
13	A	80 per cent alcohol precipitate ≏10	11	14	170.0	167.1	61.8	−4	34.9	−3	30.1	57.5
14	A	Chloroform extract ≏10	12	11	147.3	133.7	109.4	70	63.0	75	21.8	35.0
15	A	80 per cent alcohol residue ≏10	14	11	164.7	149.2	57.8	−10	29.9	−17	30.0	51.4
16	A	None (negative control)	8	16	162.1	148.2	64.4		36.1		27.2	47.1
17	A	Testosterone acetate *	9	15	155.8	151.9	751.1	1066	546.7	1414	20.9	30.7
18	A	Tesosterone acetate and estrone **	10	14	134.9	117.4	775.0	1103	526.4	1358	15.6	25.5

* Testosterone acetate was added 18 mg. per kg. of diet.
** Estrone was added, 3.6 mg. per kg. of diet.

of table 1. They show that the feces from the cows markedly stimulated comb growth in the chicks on both of the basal diets, and the feces of the heifers also stimulated comb growth but to a lesser degree; whereas the feces of the bulls had no apparent effect. The testes and ovaries of the chicks that received the basal diets supplemented with feces from cows or heifers weighed less than those of the chicks that received either the unsupplemented basal diets or those of the chicks that received a basal diet supplemented with the feces of bulls.

Experiment 2. In the first experiment the stimulating effect of the feces of cows and heifers on comb growth and its depressing effect on the gonads strongly suggested that the effect was produced by an androgenic substance. In the second experiment the effect of various fractions obtained from the feces of cows was studied.

A crude alcohol extract was prepared by adding 2 kg. of dried feces to 20 liters of 50 to 60 per cent alcohol, stirring thoroughly and allowing the mixture to stand for 48 hours at room temperature. The liquid was drawn off through a Büchner funnel. This solution was brought to a concentration of 80 per cent alcohol. A

precipitate formed that was fed to *lot 13*. The 80 per cent alcohol solution was concentrated in a vacuum still, boiled with concentrated HCl for 15 minutes and then extracted by shaking with chloroform. The chloroform extract was fed to *lot 14*. The residue was fed to *lot 15*.

Testosterone acetate was added to the diet of *lot 17* to determine the effectiveness of this androgen when incorporated in the diet, and to compare its effect with that of the active factor in the feces of cows. The estrogenic principle,[2] estrone, was combined with testosterone acetate and fed to *lot 18* to determine whether or not the response to the androgen might be influenced by the presence of estrogenic material. Both testosterone acetate and estrone were first dissolved in alcohol and then thoroughly mixed with the basal diet. Testosterone acetate was given in the proportion of 18 mg. to a kg. of basal diet; while 3.6 mg. of estrone was given per kg. of basal diet. A total of 315 mg. of the testosterone acetate was given to each

TABLE 2. SUPPLEMENTS TO THE DIET, AVERAGE DIMENSIONS OF COMBS OF ALL CHICKS, AND AVERAGE LIVE WEIGHT, COMB WEIGHT AND TESTES WEIGHT OF THE FIVE LARGEST CHICKS FROM EACH LOT IN THE THIRD EXPERIMENT

Lot No.	Basal Diet	Additions to Basal Diet		Average Dimensions of Combs of All Birds at 4 weeks		Averages for the 5 Largest Males at 4 Weeks		
						Live wt.	Comb wt.	Testes wt.
			%	Number	mm.	gm.	mg.	mg.
22	A	None, negative control		24	16.7×5.1	181.2	171.8	54.0
19	A	Cow feces (without pasture, new)	10	24	27.6×11.2	214.6	685.6	26.8
20	A	50 per cent alcohol extract	=20	24	27.3×10.8	215.0	693.3	31.3
21	A	Cow feces (without pasture, old)	10	23	17.7× 5.3	235.4	134.4	63.6
23	A	Cow feces (without pasture, new)	10	28	27.2×10.9	208.0	774.9	29.5
24	A	Water extract	=20	29	17.9× 5.9	231.8	171.4	70.2

of the two lots of 25 birds, and in addition a total of 63 mg. of estrone was given to the birds of *lot 18*. One lot of birds (*lot 12*) was fed a diet supplemented with feces from cows without access to pasture.

The results of this experiment are summarized in the second section of table 1. Marked comb growth occurred in the lots receiving *a*) feces from cows with access to pasture, *b*) the chloroform extract, *c*) testosterone acetate, and *d*), testosterone acetate in combination with estrogenic substance. In the remaining lots of birds there was no noticeable stimulating effect on the growth of the comb or depressing effect on the development of the gonads.

Since the feces from cows without access to pasture failed to stimulate comb growth it was presumed that the diet of the animals may have influenced the excretion of the active factor. However, these feces were dried for a longer time and at a higher temperature than the feces from pasture-fed cows and it is probable that the active principle was destroyed.

The average weight of the combs of both males and females receiving testosterone acetate in combination with the estrogenic material was as great as those of birds receiving the androgen alone. The alcohol precipitate had no effect on comb growth. The chloroform extract of a quantity of cow feces equivalent to that given to the chicks of *lot 11* was not so effective as the dried feces, and it is presumed that all of the active material was not extracted.

[2] The estrone used (Amniotin) was supplied by E. R. Squibb & Sons, New York City.

Experiment 3. This experiment was conducted primarily to re-investigate the effects of feces from cows without access to pasture. A sample of feces was obtained from the same stall-fed cows from which material had been collected in the previous experiment. In this case, however, the feces were dried at 45° C. in 24 hours. The effect of this supplement was compared with that of the feces of stall-fed cows which had been found ineffective previously, and also with both an aqueous and a 50 per cent alcohol extract of material from the same source.

To compare the effectiveness of the several preparations without killing the birds, the comb dimensions of all birds were obtained at 4 weeks. At this time the 5 largest males of each lot were killed and the comb and testes weights obtained. This procedure was resorted to in order to select small comparable samples from each lot and, at the same time, leave the sampled lots comparable. The results are summarized in table 2. The new batch of feces from the cows without access to pasture, dried for a shorter time and at a lower temperature than the first batch used in *experiment* 2, stimulated comb growth to approximately the same degree as that observed earlier when feces from cows with access to pasture were fed. The birds receiving the aqueous extract were heavier than birds receiving only the basal diet, but the combs showed no signs of stimulation. The alcohol extract of a quantity of feces equivalent to 20 per cent of the diet gave approximately the same degree of stimulation as the 10 per cent of dried feces supplement, indicating an incomplete extraction.

TABLE 3. EFFECT OF CHLOROFORM EXTRACT OF COW FECES, TESTOSTERONE ACETATE, AND ESTRONE ON COMB GROWTH

Substance	Total Dosage	Number of Females	Average Final Weight	Average Comb Weight	Per Cent Increase in Comb Weight over Controls
			gm.	mg.	
No treatment		9[1]	163.0	48.9	
Chloroform extract of cow feces	≏ 1 gm.	10	176.8	100.1	104
Chloroform extract of cow feces	≏ 5 gm.	9	171.7	98.7	102
Chloroform extract of cow feces	≏ 10 gm.	8	158.7	104.9	114
Testosterone acetate	1 μg.	10	171.1	65.9	35
Testosterone acetate	2 μg.	8	173.0	72.7	49
Testosterone acetate	8 μg.	7	176.4	93.9	92
Estrone	2.5 mg.	10	179.0	45.1	

[1] Controls injected with 0.05 cc. Mazola oil daily.

Assays of extracts of feces from cows. General confirmation of the results described in the above experiments was obtained by other tests conducted simultaneously. Some of the same 80 per cent alcohol precipitate which was fed to *lot* 13 in *experiment* 2 was administered orally to 6 male chicks for 4 weeks in daily doses equivalent to 2 gm. of dry feces. At autopsy on the day following the last treatment, there was no significant difference between the average comb and testes weights of the 6 treated and 6 control birds. The average comb weights were 144.6 and 154.3 mg. for control and experimental birds, respectively. The average weight of the pairs of testes of the controls was 54.9 as compared with 53.8 mg. in the case of the treated birds.

The supernatant material from the 80 per cent alcohol precipitation (described under *experiment* 2) was extracted for both male and female sex hormones. The alcohol was evaporated and the residue extracted by a method (modification of method de-

scribed by Callow et al., 5) routinely employed in extracting sex hormones from urine. The procedure consisted of separate hydrolysis for 15 minutes with concentrated HCl (10 per cent by volume), continuous extraction with chloroform and subsequent fractionation. As was subsequently shown by tests with immature mice, the androgen fraction was not completely free of estrogens.

An attempted assay for androgenic activity was made with female White Leghorn chicks that were 11 days old. The weight of the selected assay chicks ranged from 85 to 100 gm. and a further selection was made for uniformity in comb size. Ten female chicks were used at three different dose levels. The androgen fraction was taken up in Mazola oil, and one-tenth of each total dose (in 0.05 cc. of oil) was injected daily for 10 days into the base of the comb. The results of these tests, together with the response of the assay chicks to testosterone acetate and the estrogenic principle of estrone are presented in table 3.

The average weight of the combs of assay birds receiving the chloroform extract (androgen fraction) equivalent to 1 gm. of dried feces was slightly higher than that of birds receiving a total of 8 micrograms of testosterone acetate. Since it was observed in *experiment* 3 that only about one-half of the active material was extracted with 50 per cent alcohol, it is probable that 1.0 gm. of dry feces has the activity of 16 μg. of testosterone acetate. The larger doses of the chloroform extract did not produce a proportionate increase in comb size. The average weight of the combs of chicks injected with the estrogenic preparation was slightly less than that of the average comb weight of controls.

Tests for estrogenic activity in both the estrogen and androgen fractions were made by using the uterine response of the immature mouse (6). The desired quantities of the respective fractions were taken up in 0.3 cc. of Mazola oil, and the mice were injected once daily (0.1 cc.) for 3 days and autopsied 24 hours after the final injection. An 150 per cent increase over the average weight of uteri of control mice was elicited by a dose of the female sex hormone fraction equivalent to 5 gm. of dried feces and by a dose of androgen fraction equivalent to 6 gm.

DISCUSSION

The failure of any of the preparations used in these experiments to stimulate gonadal development eliminates the possibility of the active factor being a gonadotropic substance. On the other hand, the marked stimulation of comb growth in both male and female chicks and the depressing effect on gonadal development by both dried cow feces and chloroform extracts of this material strongly indicates that the active factor is an androgenic substance. The fact that cows, both with and without access to pasture, excrete the active principle in similar concentrations indicates that the androgen is not derived from the diet.

It is of especial interest, as shown in *experiment* 1, that feces from bulls were entirely without effect, whereas the feces from pregnant cows, as well as from unbred heifers, had marked androgenic effects. This seemingly paradoxical situation in cattle would appear to be the contrasting parallel of that reported for the horse in which the stallion excretes, via the urine, large quantities of female sex hormone (7).

The content of estrogenic substances in the urine of cows in various stages of gestation has been reported by Turner et al. (8). The present study indicates that some estrogenic material is also excreted in the feces of cows. The possibility of the presence of this factor influencing the comb-growth response should be taken into consideration in these experiments in which unfractionated or incompletely frac-

tionated extracts of feces from cows were used. Kosin and Munro (9), however, have recently reported that when a combination of testosterone propionate and estradiol dipropionate (2 mg. each) was injected into chicks there was no marked evidence of either synergism or antagonism of the action of the male and female hormones.

From a practical standpoint, it would be of considerable importance to identify the androgen present in feces of cows. The present methods of preparing synthetic androgenic substances for clinical use are expensive. In view of the androgenic potency of cow feces and its relative effectiveness upon oral administration, the practicability of using this material as a source of androgenic substance should be investigated.

SUMMARY

The feces of cows in various stages of gestation and of unbred heifers, when dried and fed to chicks as a supplement to their diet, stimulated comb growth. The development of the testes and the ovaries was retarded when chicks were fed material containing the active factor. These effects indicate the presence of one or more androgenic principles.

Both alcohol and chloroform extracts of cow feces possessed androgenic activity. A precipitate obtained by increasing to 80 per cent the alcohol concentration of a 50 to 60 per cent alcohol extract of feces was without effect. A chloroform extract of 1.0 gm. of dried cow feces had at least, and probably somewhat more, androgenic activity than 16 micrograms of testosterone acetate.

Feces from mature bulls were without effect on either comb growth or gonadal development.

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CORNIFICATION OF BALANO-PREPUTIAL EPITHELIUM IN NORMAL RATS AND IN CASTRATED RATS TREATED WITH TESTOSTERONE PROPIONATE[1]

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DURING the course of a search for a tissue which might show good growth response to local application of androgen, and therefore prove valuable in bioassay, the interesting phenomenon of cornification of the balano-preputial epithelium in the rat was encountered. This cornifying process, leading to a cleavage in the epithelium which forms the stratified squamous lining of the prepuce, and the cornified covering of the glans was described in 1866 by Schweigger-Seidel (1) and more recently by Deibert (2) and Hunter (3). According to Deibert the process may begin in the human fetus as early as the sixth month and although the separation is not usually complete at birth, it may be sufficient by the 10th day to allow retraction of the prepuce. Incomplete separation or persisting adhesions in these membranes have been recognized as phimoses of varying types and almost yearly new procedures for their correction have been proposed. We are not aware that anyone has resorted to the local application of male hormone. And yet an observation made in cats by Retterer (4) in 1887 and recalled by him and Lelievre in reports of further experimentation in 1912 (5) clearly connected this penile phenomenon with a testicular secretion.

In 1933, Wiesner (6), in a brief report on the effects of androgen on the penis and clitoris described the epithelial covering of the glans penis in normal and castrated rats, as well as in castrated rats treated with androgen. He attributed the peculiar cornification of the penile epithelium to the male hormone and related this process to that occurring in the vagina in response to estrogen. More extensive studies of a similar nature were made later by Raynaud and Lacassagne (7) in mice. Our own findings in the Long-Evans strain of rats are completely in accord with those of Raynaud and Lacassagne, especially with regard to the time of cornification and cleavage. Lacassagne and Raynaud not only induced cleavage of the balano-preputial epithelium in castrated mice by injecting testosterone acetate, but obtained comparable results in the clitoris of mice treated with the same hormone (8). Korenchevsky et al. (9) had also reported stimulation of "the clitoris-like organ' in ovariectomized rats treated with androstanedione and stated that it appeared 'like a short rudimentary penis with a praeputium-like folding of skin over it.'

Greene and Wigodsky (10) reported that by applying 75μg. of testosterone locally to the penis for 22 days they were able to induce in rats castrated at 24 days of age, a growth of penis 'approximately of the same order as that of normal untreated rats.'

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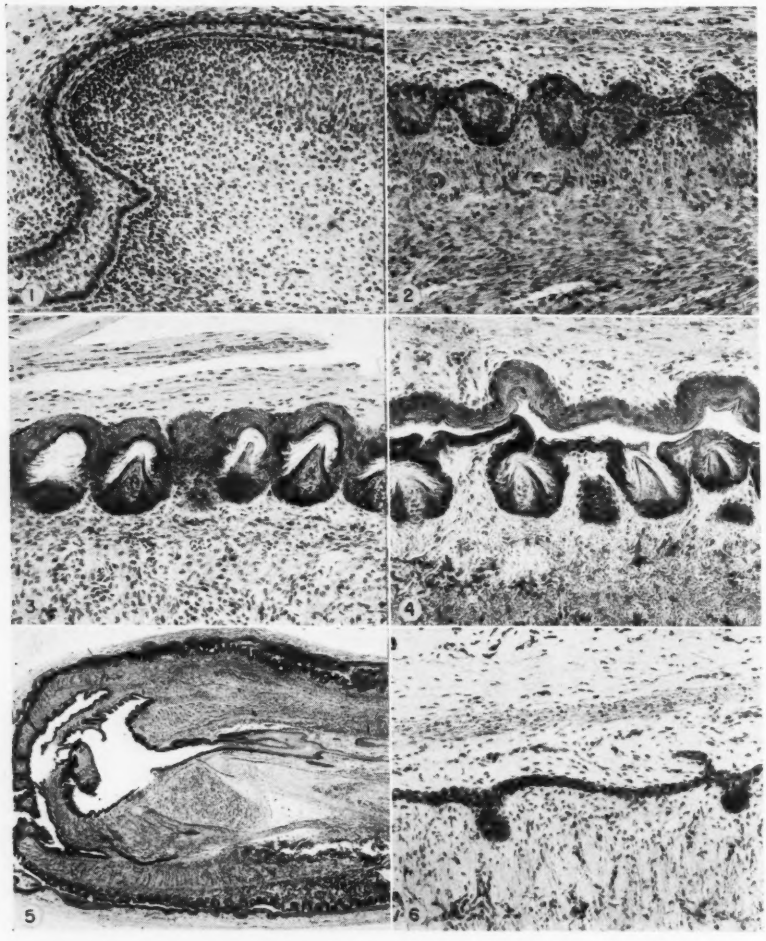

All figures ×92 except Fig. 5 which is 12×.

Fig. 1. Penis of 1 day old, normal rat. Note inner and outer germinal layers.

Fig. 2. Penis of 24 day old, normal rat. Papillae formed, but not cornified.

Fig. 3. Penis of 45 day old, normal rat. Papillae cornified and glans-prepuce separation beginning.

Fig. 4. Penis of 52 day old, normal rat. Glans-prepuce separation complete.

Fig. 5. Penis of 52 day old, normal rat. From same animal as fig. 4, ×12.

Fig. 6. Penis of adult rat, 30 days after castration. Glans-prepuce fusion and disappearance of cornified papillae.

These authors made no mention of the glans-prepuce separation in their normal or treated animals which were 47 days old at necropsy. They claimed to have drawn back the prepuce of their 24-day-old rats in order to apply hormone or oil directly to the penis, but it seems likely that except for exposing the tip of the glans, they merely retracted the outer skin fold rather than the praeputial fold which adheres to the glans in our rats and in mice (7) until they are approximately 47 days old. That the

separation of the prepuce from the glans may be induced at an earlier age by androgen treatment will be shown in the following report.

PROCEDURE

Our investigation was made in three parts. *a*), Sections of penes for histologic study were obtained from 8 groups of 3 rats killed on *days* 1, 8, 16, 24, 31, 38, 45 and 52. *b*), Twenty-nine rats were examined daily beginning at *day* 40, in order to determine the variation in the time of separation of the prepuce from the glans. *c*), Groups of 3 rats castrated at *day* 26 were injected subcutaneously with varying dosages of testosterone propionate (12.5–600 μg. daily) in 0.1 cc. of sesame oil for different periods of time in order to determine the minimal dose required to accomplish the same cornification and cleavage as occurs in the normal rat penis.

RESULTS

Histologic study of the balano-preputial cleavage. Histologic study of the new-born rat penis revealed that proliferation of the epithelium, in which the preputial 'moat' forms later through cleavage had already begun. As shown in figure 1, an outer and inner layer of germinal cells are proliferating at this time. Between these two layers

TABLE 1. NUMBER OF DAILY INJECTIONS OF VARYING LEVELS OF TESTOSTERONE PROPIONATE REQUIRED TO EFFECT SEPARATION OF THE PREPUCE FROM THE GLANS PENIS IN RATS CASTRATED AT 26 DAYS OF AGE AND INJECTED IMMEDIATELY THEREAFTER

Rat	Dose, mg. in 0.1 cc.	No. of Daily Injections	Total Dose, mg.	Rat	Dose, mg. in 0.1 cc.	No. of Daily Injections	Total Dose, mg.
1	0.6	7	4.2	10	0.05	11	0.55
2	0.6	7	4.2	11	0.05	9	0.45
3	0.6	6	3.6	12	0.05	8	0.40
4	0.4	7	2.8	13	0.025	13	0.32
5	0.4	7	2.8	14	0.025	11	0.27
6	0.4	6	2.4	15	0.025	10	0.25
7	0.2	8	1.6	16	0.012	24	0.29
8	0.2	7	1.4	17	0.012	21	0.25
9	0.2	7	1.4	18	0.012	20	0.24
10	0.1	9	0.9	19	Uninjected castrates, showed no separation during 3 weeks of observation		
11	0.1	9	0.9	20			
12	0.1	8	0.8	21			

and contributed to by both, may be seen an intermediate zone of degenerating or squamous cells. Very little qualitative difference was found in the glans-prepuce epithelium until the 16th and especially the 24th days. By this time, as may be seen in figure 2, the outer epithelium which eventually forms the preputial-lining and the inner epithelium which forms the covering of the glans had proliferated considerably with the latter already forming regularly-spaced papillae by a process somewhat comparable to that observed in hair or scale development. The term cornification is especially applicable to the process that occurs in these papillae from the 31st day on. Figures 3 and 4, representing the 45th and 52nd day stages, respectively, show the small 'horns' that form in the epithelium covering the glans. These cornified papillae are barely visible to the naked eye and cover the surface of the glans of the sexually mature rat. A process of 'soft' keratinization somewhat similar to that occurring for the first time in the vaginae of rats of approximately the same age (35–45 days) takes place in the epithelium between each papilla and in that lining the prepuce.

As shown in figures 4 and 5, the inner and outer epithelial layers have split apart by *day* 52, and this allows the prepuce to be retracted completely exposing the glans. During the period between the fourth and seventh weeks approximately, only the tip of the glans could be exposed by drawing back the exterior skin fold. Figure 6 shows the atrophic balano-preputial epithelium which has almost completely fused again 30 days after castration.

On the time of balano-preputial cleavage in Long-Evans strain of rats. In the group of 29 rats examined daily to determine the time of glans-prepuce separation it was found that this occurred in the majority by the 47th day. One rat showed separation on *day* 44, two on 45, three on 46, ten on 47, three on 48, four on 49, two on 50, two on 51, one on 54 and one on 55. No attempt was made to tear the prepuce back from the glans, and so it was found that in some rats within a day of the first signs of retractability, separation was complete, whereas in others this was so only after 2 to 5 days. In a second group of 29 rats examined for the first time on *day* 50, twenty-three showed partial or complete separation and in the remaining 6, separation occurred on the following day. It may be of interest to recall that during the period when the penis is undergoing its final alterations for service as a copulatory organ the sperm have just become motile and available in the epididymis.

Balano-preputial cleavage induced in castrated rats with testosterone propionate. The results obtained on treating rats castrated on *day* 25 with testosterone propionate are given in table 1. It may be seen that preputial separation occurred after one week of daily injections, amounting to 0.2, 0.4 or 0.6 mg. This suggests that maximal rapidity of response was obtained with the 0.2-mg. dose as well as with the higher dosages. These higher levels of hormone dosage accomplished in one week the same penile changes in castrates as normally require 3 weeks in the intact rat. It is interesting to note that the lowest daily level of hormone used (0.012 mg.) had to be administered for 3 weeks which happens to be precisely the length of time that would have elapsed (before cleavage occurred) had the animals not been castrated. Thus it may be said that during the 3-week period (fourth to seventh weeks) when normal rats are gradually attaining sexual maturity, they are probably not exposed to more than a mean daily equivalent of 12 micrograms of testosterone propionate. Approximately the same total dose of hormone (0.25–0.3 mg.) was required whether given in daily doses of 25 or 12μg., but the higher daily dosage brought about preputial separation in 1.5 weeks as compared to 3 weeks of the lower dose.

SUMMARY

Histologic studies were made of the penes of 24 normal, Long-Evans rats killed in groups of 3 at weekly intervals from *day* 1 to *day* 52. It was found that the epithelium, from which arises the preputial lining and the glans covering, begins to cornify by the 31st day, or earlier. In the 45-day-old animals cornification was well advanced and cleavage to form two epithelial surfaces had already begun. This separation was found to be completed in the 52-day-old animals.

In a group of 29 Long-Evans rats examined daily to determine when the prepuce separated from the glans penis, the majority (16 in number) showed retractable prepuces by *day* 47 and the remaining by *day* 55.

Daily subcutaneous doses of 0.2, 0.4 and 0.6 mg. of testosterone propionate in sesame oil given to rats castrated at *day* 26 and injected immediately thereafter, caused preputial separation in one week. The number of daily doses of 12, 25, 50 and 100 μg. of the hormone required to accomplish this separation varied inversely with the

amount of hormone per dose. The 12-μg. dose had to be given for 3 weeks, the time of cleavage in these rats corresponding to that in the normal intact controls.

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METHOD FOR OBTAINING UTERINE FLUID FROM THE MONKEY: EFFECT OF PILOCARPINE, ATROPINE, PHYSIOLOGICAL SALT SOLUTION AND ADRENALIN

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THE PURPOSE of this paper is to describe a method for collecting uterine fluid from the monkey in chronic experiments and to record preliminary observations in its use.

In 1940 van Wagenen and Morse (1) reported a technique for the creation of a permanent utero-abdominal fistula in the monkey and pointed out that an easy entrance into the uterine cavity was thus readily made available. It seemed worth while to determine whether a similar preparation could be used to obtain samples of uterine secretions for study at various times in the menstrual cycle. For this purpose four *Macacus rhesus* monkeys from the Carnegie colony were selected for operation. It was found desirable to choose monkeys with well-developed and freely movable uteri and with a record of normal menses. Observations were made from February to the end of April at which time the cycles of the selected monkeys began to show the seasonal irregularity that generally precedes the summer amenorrhea.

Operative technique. Approximately the same technique was used to create a utero-abdominal fistula as that described by van Wagenen and Morse. A midline incision was made, and the round ligaments were cut at their lateral attachments. These ligaments were then threaded subcutaneously over a part of each rectus muscle and passed down through a stab wound in the muscle bellies and peritoneum, to be sutured back against the fundus within the peritoneal cavity. After closure of the peritoneum and fascia at both ends of the incision, the fundus protruded out of the abdominal cavity into the center of the wound. A few stitches from fundus to peritoneum held the uterus firmly in this position. Excision of a core of myometrium with endometrium at its tip gave a satisfactory opening into the cavity of the uterus. The skin edges on either side of the fistula were brought down into the crater in the myometrium and fixed with fine silk to form a dimple at the orifice. No dressing was used, and the wounds were generally healed in 2 weeks.

A third membrane of scar epithelium almost invariably formed over the fistula opening, as reported by van Wagenen and Morse. If this membrane was opened and the underlying tract dilated every 3 or 4 days, over the course of several months there was little tendency for the tract to close down. With this frequent, gentle instrumentation a minimum of bleeding resulted.

A fine uterine biopsy curette was made, similar to that described by Meigs for use in the human female (2) but on a smaller scale. Fragments of endometrium could be obtained with this instrument through the fistula. These specimens, after fixing and staining, were studied to check the records of daily rectal palpation of the ovaries for determining the occurrence of ovulation.

Collection of uterine fluid. Three observations were generally made during a menstrual

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cycle, *a*) at the end of the first week after onset of menstruation, *b*), approximately at the time of ovulation, and c), a few days before the next expected menses.

In order to observe the rate of flow of uterine fluid from the fistula under reasonably basal conditions over a period of several hours, the monkey was kept under light anesthesia with nembutal, subcutaneously, avertin, rectally, or pentothal sodium, intravenously. Pentothal proved the most satisfactory of these. For induction of anesthesia, about 1 cc. per kg. of a 2.5 per cent solution was used; for its maintenance, about 10 cc. by vein per hour of a 0.2 per cent solution was generally sufficient.

The animal was placed on her side on the table and the perforated end of a No. 8 catheter, previously filled with mineral oil, was gently worked through the fistula into the uterine cavity. The other end of the catheter was connected to the barrel of a tuberculin syringe (graduated in hundredths of a cc.) for measuring the output of uterine fluid. The lower end

TABLE 1. RATE OF FORMATION OF UTERINE FLUID: NORMAL, OR CONTROL READINGS

Day of Cycle	6	7	9	14	16	21	25	26	27
Month One	.168			.152	ov.				Menses
Month Two			.08	ov.	.152		.140	Menses	
Month Three		.092				.141			

Monkey 166. Average secretion in cc. per hour in 8 experiments on different days of consecutive months. 'Ov' indicates probable date of ovulation as determined by daily rectal palpation. In *month three* there was no evidence of ovulation and the menses were delayed for 50 days.

of the syringe barrel was fixed arbitrarily at a level 15 cm. below that of the fistula. This relationship was used in all of the experiments to insure a constant small amount of subatmospheric pressure equal to the weight of the 15 cm. column of mineral oil in the catheter. The system was rendered air-tight by fixing the catheter securely to the abdominal wall with wisps of cotton, glued down with collodion. A small, glass connecting tube was interposed between sections of the catheter close to the abdomen to detect any seepage of air into the system and to observe the character of the fluid. Changes in the oil level in the syringe barrel recorded the displacement of oil in the proximal end of the catheter by fluid from the uterine cavity.

Blood pressure changes were followed with a mercury manometer, connected with an infant's size blood pressure cuff wrapped around the upper arm of the animal.

Intravenous solutions were given through a needle fixed in a the left saphenous vein, from calibrated burettes connected through a two-way stop-cock. A small glass air-chamber in the rubber tubing was found helpful in controlling the flow, drop by drop, to as little as 20 cc. per hour.

Readings of the oil level in the syringe barrel and blood pressure, pulse rate and respirations were recorded every 15 minutes throughout each experiment.

Basal rates of flow. We have not had an opportunity to measure the rates of flow of uterine fluid sufficiently to permit detection of a cyclic variation. The control, or basal readings, before any pharmacological procedures, however, are shown in table 1. No correlation was possible between these data and the respective menstrual cycles, especially with regard to the occurrence or absence of ovulation. The third cycle listed in table 1 was anovulatory, the first two were ovulatory, as determined by rectal palpation.

In a castrated monkey, given 1,000 I.U. of estrogen[2] daily for 12 days, the sex skin was a deep red color. On the 13th day of the treatment, .043 cc. of uterine fluid per hour was obtained. Five mg. of progesterone per day for 8 more days was then given; the next day, the rate of flow of uterine fluid was .076 cc. Clearly, further observa-

[2] The estrogen (Amniotin) was supplied by E. R. Squibb & Son, New Brunswick, N. J.

tions will have to be made to determine the effect of different dosage levels of the hormones, singly and in combination, on the rate of formation of uterine fluid.

When a foreign body such as a soft rubber catheter is introduced into any hollow organ it can be assumed that the irritation thus produced will cause a certain amount of discharge. In the present experiment is is believed that the amount of irritative discharge from the walls of the fistula was negligible, since this tract was well epithelialized within a few weeks after operation. By probing and measuring the depth of the fistula, it was possible to introduce the catheter in such a way that the collecting holes at its tip lay entirely within the the uterine cavity. Attempts to siphon off fluid from the external opening without entering the tract at all were unsuccessful. It is impossible to determine how much of the collected fluid is directly due to the irritation of the inlying catheter tip, but the total of 1.25 cc. in 10 hours in the experiment shown in figure 1 seems to be far more than one could reasonably

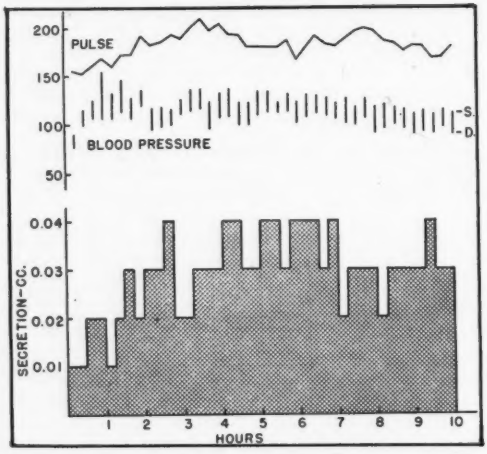


Fig. 1. Flow of uterine secretion in relation to pulse and blood pressure.

ascribe to this cause alone. In some cases this flow amounted to more than 0.25 cc. per hour. The rate of flow, recorded every 15 minutes, was probably more constant than it appears to be in figure 1. The seeming variations between readings were unavoidable, since it was not possible to measure the bottom of the meniscus of oil in the syringe more accurately than to the nearest 0.01 cc. mark. The constancy of the rate of flow of fluid (in the absence of some experimental procedure to modify it) collected over 9 or 10 hours argues more for a physiologic than for a pathologic process.

The total amount of fluid collected in any one 15-minute period could have absolute significance only if it were corrected by deducting an 'x' factor, the amount due to irritation alone. This factor is undeterminable but it must be assumed to be relatively constant for all of the experiments, since the apparatus and procedure were identical in all cases. One may conclude, therefore, that although the quantity of fluid collected may not precisely represent normal secretion without an irritant, yet a comparison of rates of secretion at different times in the cycle may ultimately yield significant correlations. It is generally recognized that as much must be said for other techniques of study, such as the recording of uterine motility by an intra-uterine balloon.

It may be questioned whether the anesthetic agent itself might not influence the formation of uterine fluid. The use of ether was entirely avoided. Only the smallest amount of nembutal or pentothal was given that would keep the animal quiet, without struggling. The corneal reflex was often present through most of the experiments. It was only when concentrated solutions of the anesthetic were used for induction, causing a temporary de-

pression of blood pressure and pulse that there appeared to be an associated depression in secretion rate.

The character of the fluid could be observed through the glass connecting tube close to the fistula. This fluid was almost always clear, yellow-pink and generally acellular through the first 4 or 5 hours. At about that time there would usually appear in the glass tube small amounts of less translucent, mucoid material, with fine streaks of fresh blood, alternating with the previously described yellow-pink fluid; this appearance was maintained throughout the remainder of the experiment. Occasionally a little frank blood would be seen. The possibility that the mucoid material may have been cervical or endocervical secretions sucked back into the uterus cannot be overlooked in a qualitative analysis of the fluid so collected, unless the cervical glands are destroyed previously or otherwise are isolated from the uterine cavity. This precaution was not taken in the present study but the amount of mucoid material was not sufficient to affect materially the rates recorded in these experiments. It is possible that the unusual anatomy of the cervix, with a colliculus acting as a sort of valve, may partially obstruct the retrograde withdrawal of secretions from glands of the cervix uteri in the macaque.

TABLE 2. EFFECT OF PILOCARPINE ON UTERINE SECRETION COMPARED WITH OTHER PHYSIOLOGICAL RESPONSES

Pilocarpine, dose	Day of Cycle	Figure No.	Uterine Fluid, Change in Rate of Flow	Effect on Other Physiological Mechanisms							
				Vascular		Secretory			Smooth Muscle		
				Blood pressure	Heart rate	Salivation	Perspiration	Lacrimation	Defecation	Urination	Vomiting
0.05 mg. per kg.	6		o			o	o	o	o	o	o
0.5 mg. per kg.	26	I	o	o	?	+	+	o	o	o	o
1.5 mg. per kg.	26	II	o	−	?	++	++	+	o	o	o
3.0 mg. per kg.	15	III	−−	−−	−	+++	+++	++	++	++	++
5.0 mg. per kg.	9	IV	−−−		−−−	+++	+++	+++	o	++	o

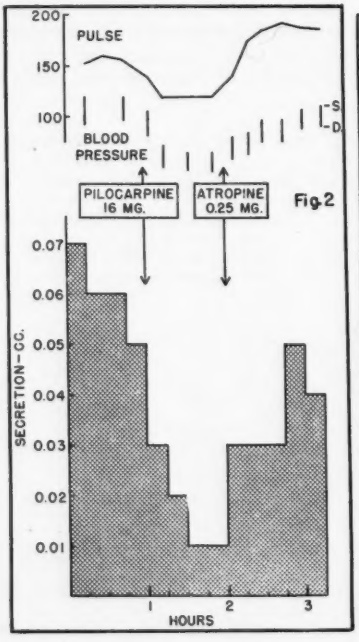
One may, therefore, say that *a*), the effect of irritation on the flow of uterine fluid is immaterial when comparative rates, and not absolute quantities of fluid, are being considered; *b*) that maintenance doses of the anesthetic had no observable influence; *c*) that the amount of secretion of possible cervical origin in the fluid was negligible. It therefore follows that the rate of flow here reported probably does represent a reflection of the fluid formed in an unoperated uterus. The latter statement, however, demands the following qualification. Since, in the intact animal, there is no mechanism exerting a constant sub-atmospheric pressure to withdraw the fluid as it is formed, the rate of flow here described should be interpreted as a record of the potentiality of the endometrium to form fluid. The direct or indirect effect, if any, which rhythmic contractions on the myometrium may have cannot be stated at this time. A study of electrolytes, glycogen content and other characteristics of uterine secretions, making use of this method, must take into account factors which influence this rate of flow. The importance of several such factors is suggested in the experiments reported below.

Action of Drugs and Physiological Salt Solution

Since the total output of uterine fluid in the monkey was small, hardly exceeding 1 cc. in several hours, it seemed desirable to explore at the outset of the experiment the possibilities of altering the rate of secretion by the action of certain drugs. In

1930 Rocco de Marco (3) reported that the flow from a uterine fistula in the bitch during heat could be increased fourfold by subcutaneous injections of pilocarpine, while no effect on the rate of flow could be observed after injections of atropine, eserine or pituitrin. Accordingly, an attempt was made to confirm this finding for the monkey by giving varying doses of pilocarpine at, or near the time of ovulation and early and late in the cycle as well. The effects of atropine, of intravenous saline and of adrenalin were also studied.

Pilocarpine. The effect of increasing doses of pilocarpine on the flow of uterine fluid is shown in table 2, together with the vascular, secretory and certain smooth



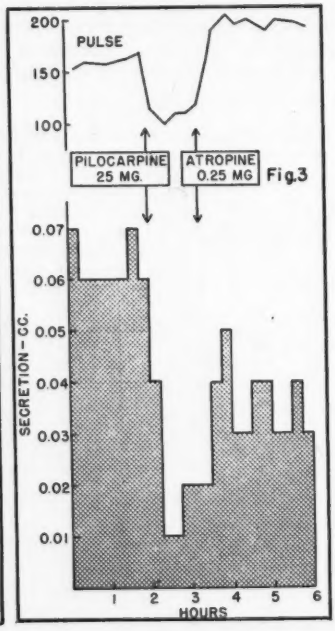


Fig. 2. Flow of uterine secretion. A dose of 16 mg. of pilocarpine caused a decrease in secretion rate, blood pressure and pulse rate. These returned toward previous levels after atropine.

Fig. 3. Flow of uterine secretion. A dose of 25 mg. of pilocarpine caused an abrupt decline in secretion rate. This returned toward its previous level after atropine.

muscle effects at the various dosage levels. It is immediately apparent that the flow of fluid from the uterus was never increased in any of the experiments. The smallest amount of the drug to produce visible effects (salivation, perspiration) was 0.5 mg. per kg., or a total of 2.7 mg., subcutaneously. This had no discernible effect on the rate of formation of uterine fluid. A dose of 1.5 mg. per kg., (totalling 7 mg.), caused a slight drop in blood pressure as well. When 3.0 mg. per kg., or 16 mg. in all, was used (fig. 2) the effects were marked within 10 minutes following the injection, and the flow of uterine fluid decreased within 45 minutes from a level of 0.07 cc. to 0.01 cc. per 15 minutes. The animal showed profound prostration, with systolic blood pressure well below 100 mm. Hg at this dosage level. Atropine, 0.25 mg. subcutane-

ously, was given in a short time because the monkey's condition appeared critical. After administration of the atropine the blood pressure, pulse rate and uterine secretion rate rose toward the levels recorded before injection of pilocarpine. A similar, but even more abrupt drop in secretion rate occurred after 5 mg. of pilocarpine per kg. was given (25 mg. in all), as shown in figure 3. Again the level of uterine secretion recovered somewhat after 0.25 mg. of atropine.

From these observations it is concluded that the stimulating effect of pilocarpine on uterine fluid secretion which was noted by de Marco in the bitch can not be confirmed in the monkey. On the contrary, injections of 3 mg. or more per kg. depressed the flow of uterine fluid, while smaller doses had no measurable effect on this

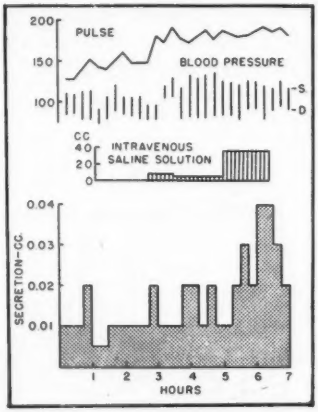


Fig. 4. INCREASE IN FLOW OF UTERINE SECRETION AFTER RAPID SALINE INFUSION. This was in a castrated monkey which had been given daily estrogen for the 2 previous weeks.

mechanism. The physiological basis for this difference in response to pilocarpine in the bitch and monkey, respectively, requires further study.

Atropine. A single dose of 0.25 mg. of atropine subcutaneously had no observable effect on the rate of uterine fluid formation, blood pressure or pulse rate. Similarly, the same amount of atropine had no effect after as little as 0.5 mg. of pilocarpine had been given. Increases in the rate of flow of uterine fluid following atropine occurred only after giving large doses of pilocarpine (fig. 2, 3). Only when such doses caused marked loss in fluids through sweating, lacrimation, and probably secretion into the gastro-intestinal tract as well, was atropine found to influence uterine fluid formation. These findings suggest that the action of atropine in these cases may have been that of checking further loss of body fluids and favorably affecting re-absorption of the accumulated secretions from the intestinal tract. Thus a partial restoration of total body fluid would be produced, and a partial return to the rate of uterine secretion seen before medication.

Intravenous saline. Rapid infusions of physiological salt solution were given in other experiments on the basis of the postulate outlined above that changes in the amount of body fluids and, more specifically, variations of the total blood volume influence secretion rates. Plasma administered intravenously might have been better

but it was not readily available. Figure 4 diagrammatically shows the result of this procedure. This animal had been castrated, then given 1000 I.U. of estrone daily for the 13 days previous to this experiment. When saline solution was given at a rapid rate (about 150 cc. per hour), an appreciable increase in uterine secretion was noted. The monkey was then given progesterone, 5 mg. daily for the next 8 days while the same dose of estrogen was continued. A similar increase in secretion rate was again seen after intravenous saline. Clearly, therefore, the rate of formation of uterine fluid reflected faithfully the general state of fluid balance in the body in these experiments. When fluid was lost excessively as a result of excessive doses of pilocarpine, or when

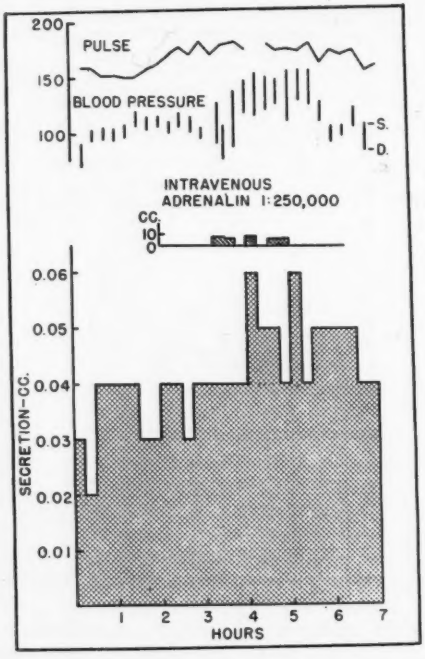


FIG. 5. INTRAVENOUS ATROPINE caused an increase in rate of uterine secretion coincidentally with a sustained increase in systolic blood pressure to over 150 mm. of Hg.

it was gained through intravenous infusion of saline, the output of fluid by the uterus fell or rose accordingly.

Adrenalin. When a solution of adrenalin chloride (1:250,000) was given slowly by intravenous drip, a slight but definite increase in the rate of fluid formation was seen (fig. 5). This increase roughly paralleled an elevation of systolic blood pressure, which was sustained at a level above 150 mm. of Hg for a 2-hour period. The same result occurred in each of 3 separate experiments.

DISCUSSION

In an analysis of the results presented above it is pertinent to inquire first into the possible significance of observed differences in the rates of flow, and second into the importance of the findings and their relation to other studies.

It was pointed out above that a variation of 0.01 cc. from one reading to the next can have no importance unless the new level is maintained over several readings. In some of the data the difference in the rate of flow of the fluid collected after medication is fairly obvious (see figures 2 and 3). In others, it is impossible to evaluate the importance of post-medication changes without resort to statistical methods. In one experiment, for instance as shown in figure 5, the readings in the second half of the experiment, after adrenalin, tended to be higher than those in the first half. In order to determine the statistical significance of the higher readings a computation was made according to Fisher's method (4, 5) for the evaluation of small samples. Table 3 summarizes the figures obtained in this calculation. According to Fisher's table of 't,' the value for P under the circumstances given is 0.001. In other words, if a great many observations were made under the same conditions there is about one chance in a thousand that one might encounter a group of higher readings similar to those of *series B*, in the second half of the experiment. The increase in the rate of secretion

TABLE 3. STATISTICAL ANALYSIS OF THE OBSERVED DIFFERENCES IN RATES OF FLOW OF THE UTERINE SECRETION

	Series A	Series B
	1st to 4th Hour	4th to 7th Hour
Number of readings	16	9
Mean for each series in hundredths of a cc.	3.62	4.88
Difference between means	1.26	
Combined mean for all readings	4.07	
Sum of all deviations^2	17.835	
Degrees of freedom	24	
Estimated σ	±0.845	
Value of 't,' $N_1=16$		
$N_2=9$	3.68	
Value for 'P,' N=24		
't'=3.68	0.001	

from the fourth hour may be regarded as significant. The question of the cause or causes of this increase, or its possible relation to adrenalin is, of course, not answered by the above computation and needs further study.

It appears possible that changes in the volume of circulating blood in the pelvis may be of primary importance in the rate of secretion of uterine fluid. This is suggested by *a*) the decrease in rate seen with depression of blood pressure and pulse rate after pilocarpine; *b*) by the reverse findings after intravenous saline, and *c*), by the effect of adrenalin. Blood pressure and pulse rate alone are, however, poor criteria of circulating blood volume, and determination of hematocrit values and bleeding experiments have not as yet been made. Too little is known about the origin of uterine fluid, and about possible changes in pelvic vascularity as the result of hormone action to allow further interpretation of these findings.

The importance of this method of collection of uterine fluid would seem to lie in its application to the qualitative chemical analysis of uterine fluid. Cyclic changes in the pH, and in the viscosity and penetrability of cervical secretions to sperm by Lamar and Delfs (6) appear to have a direct bearing on problems of human sterility. Study of similar characteristics of uterine fluid should have equal value. Studies of the glycogen content of the fluid at different phases of the cycle might prove of even greater interest. MacLeod (7) has shown that human spermatozoa are dependent entirely on utilizable carbohydrate in the medium of their environment as a source of energy for motility. Since spermatozoa can use glycogen, he suggests the possibility

that an abundance of uterine glycogen (more specifically, glycogen in the uterine fluid) may play an essential or determining rôle in nutrition of spermatozoa in their passage through the uterus to the tubes. Previous studies of endometrial glycogen during the menstrual cycle in primates have rested on analysis of crushed fresh tissue (8) or the reaction of microscopic sections to Best's carmine stain (9). Both of these methods give information concerning the glycogen *in situ* in the endometrium and, therefore, probably relate to storage and utilization. Neither method is useful, however, in showing the amount of glycogen available in the uterine fluid itself at different times in the cycle. The importance of this for fertility is shown by the work of MacLeod. The method of study herein described may yield this information. A simple modification in procedure would be necessary. Since amylase has been found in human uterine secretions (10), the fluid would have to be collected at a low temperature by surrounding the catheter and collecting receptacle with salted ice or other cooling device. In this way amylase activity could be suspended until enough fluid was obtained for microanalysis. The cervical glands should be previously destroyed as a precaution against contamination of the fluid by cervical secretions. Unforeseen circumstances have unfortunately forced a postponement of such a study in this laboratory for the present.

SUMMARY

By the use of an utero-abdominal fistula for collecting uterine fluid in the *Macacus rhesus* monkey, attempts were made to alter the rate of flow with pilocarpine, atropine, physiological salt solution and adrenalin. Pilocarpine did not cause an increase in secretion rate as previously reported for the bitch. On the contrary, large doses depressed the flow of uterine fluid. Adrenalin and intravenous saline, however, increased the rate of flow. There is some evidence that factors which influence the rate of blood flow locally or cause systemic blood volume changes may produce alterations in the rate of formation of uterine secretions. This method appears to lend itself to the further study of cyclic variations in the physical and chemical characteristics of uterine fluid in the monkey.

The author wishes to express his gratitude to Drs. G. W. Corner and S. R. M. Reynolds for their help and encouragement, and to Mr. Arthur G. Rever for skilful technical assistance in this study.

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MALE AND FEMALE MATING BEHAVIOR IN PREPUBERALLY CASTRATED FEMALE RATS TREATED WITH ANDROGENS[1]

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THAT MANY of the phenomena associated with mammalian estrus are dependent upon ovarian hormones is a well established fact. In lower mammals ovariectomy of the adult female results in cessation of cyclical changes in the vaginal epithelium, eradication of the running-activity cycle and elimination of sexually receptive behavior. Prepuberal castration prevents the appearance of these physiological and behavioral reactions. Administration of female sex hormones to the castrate female induces vaginal cornification, increased general activity and sexual receptivity.

Despite the obvious interrelationships of ovarian secretions and feminine copulatory reactions, there is some evidence to indicate that sexually receptive behavior may appear in the absence of the ovarian hormone. Male rats mounted by other males occasionally exhibit responses typical of the receptive female of their species (1, 2). Such males cannot be regarded as 'feminized,' because they display active masculine copulatory behavior which results in fertile matings. The proportion of male rats (3) or mice (4) showing feminine mating reactions, as well as the frequency of such behavior in each individual, is markedly increased by the administration of testosterone propionate. At the same time, androgen treatment results in a marked increase in the vigor of masculine copulatory performance.

It has previously been suggested that males of many vertebrate species possess neuromotor mechanisms capable of mediating the mating pattern of either sex (2). Experimental results indicate that heavy doses of androgen may increase sexual excitability to such a degree that when the stimulus situation offers no opportunity for male behavior, but does permit the execution of feminine reactions, the female pattern will appear (3, 4, 5).

Earlier investigations have established the fact that female rats exhibit masculine copulatory reactions (2, 6), and that such behavior may occur when the female is sexually receptive. However, unlike sexual receptivity, the masculine responses of the female are not dependent upon ovarian hormones and their manifestation is unaffected by castration (7). One purpose of the present study was to determine the effects of androgen administration upon male sex behavior in the castrate female rat.

The second object of these experiments was to observe the effects of androgen upon the feminine copulatory performance of castrated females. The increase of sexually receptive behavior shown by males treated with testosterone propionate suggested the possibility of obtaining similar results with females.

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MATERIALS AND METHODS

Ten female rats castrated at 21 to 27 days of age were raised in segregation and tested for the occurrence of masculine copulatory reactions between the ages of 35 and 67 days. During these tests, the results of which are reported elsewhere (7), the females were given no hormone treatment and had no contact with males. Two weeks before beginning the present investigation each female was placed in an isolation cage in which she lived for the duration of the experiment.

Sex tests were conducted on alternate days omitting Saturdays and Sundays. In each test the experimental female was placed in the observation cage (circular, 30″ in diameter) first with a sexually vigorous male for 3 minutes. In the second 3-minute period the male was replaced by a receptive female; and during the final 3 minutes of the 9-minute test the male was again put with the experimental female.[2]

During each test records were made of the male's mounting behavior and of the experimental female's masculine and feminine copulatory responses. Three types of masculine reactions were recorded for the females. *a*) The sexual clasp consists of mounting a second rat with the forepaws clasping the stimulus animal about the laterolumbar region. *b*) Palpation and pelvic thrusts includes the sexual clasp, but the forepaws are moved rapidly inward and downward repeatedly to palpate the stimulus animal; and vigorous piston-like thrusts are executed with the hind quarters. *c*), The complete pattern involves all elements described for palpation and pelvic thrusts, and in addition the copulating rat dismounts after a particularly forceful final pelvic thrust and runs or falls backward away from the stimulus animal. Females showing the complete pattern often follow this reaction by assiduously licking the genitals in a manner typical of the copulating male.

The three types of feminine sexual behavior recorded for the experimental females were: *a*), the hopping reaction, *b*), the ear-wiggling reaction, and *c*), the execution of lordosis when mounted by the male.

Each female was given 10 sex tests prior to the administration of hormone. Intramuscular injections of 1 mg. of testosterone propionate[3] were made daily except Sundays until each rat had received 14 mg. On each of the next 2 days 2.5 mg. were given; and on the day of the last injection (3 days before the final test) the amount administered was 5.0 mg. Each female received a total of 24.0 mg. over a period of 2 weeks. While hormone treatment was in progress sex tests were given on alternate days.[4]

RESULTS

Masculine copulatory reactions. Data presented in table 1 show that prior to hormone administration male sexual behavior was shown by all 10 prepuberally castrated females. The sexual clasp was exhibited by every animal and appeared in 92 of 100 tests. Palpation with pelvic thrusts, which occurred in 46 tests, was displayed by 9 females. The complete pattern was quite rare. The effects of testosterone propionate were to reduce the occurrence of the sexual clasp and to increase the frequency of the other 2 types of masculine response.

Further comparisons of masculine behavior occurring before and after androgen

[2] Males used as stimulus animals were given 3.0 mg. of testosterone propionate before tests began, and subsequent injections during the test series amounted to 5.00 mg. per male. This treatment insured that the males would continue to attempt to copulate with experimental females in the absence of receptive behavior and in spite of active resistance.

[3] Testosterone propionate (Perandren), 5 mg. per cc., was supplied by the Ciba Pharmaceutical Products, Inc., Summit, N. J.

[4] The observations herein reported were made by Miss Priscilla Rasquin whose invaluable assistance is gratefully acknowledged.

TABLE 1. EFFECTS OF TESTOSTERONE PROPIONATE UPON THE PERCENTAGE OF THE GROUP SHOWING MALE BEHAVIOR AND UPON THE PERCENTAGE OF TESTS IN WHICH SUCH BEHAVIOR APPEARED

Types of Masculine Response with Reference to Androgen Administration	Sexual Clasp		Palpation with Pelvic Thrusts		Complete Pattern	
	Before androgen administration	After androgen administration	Before androgen administration	After androgen administration	Before androgen administration	After androgen administration
Percentage of group showing response	100	100	90	100	10	90
Percentage of tests in which response appeared	92	69	46	88	1	38

treatment appear in table 2. Under the influence of testosterone propionate the frequency of all types of masculine response combined was increased 95 per cent. At the same time the absolute and relative frequency of the sexual clasp declined, and that of palpation with pelvic thrusts, and the complete pattern increased markedly.

TABLE 2. EFFECTS OF TESTOSTERONE PROPIONATE UPON THE NUMBER OF MALE RESPONSES SHOWN AND UPON THE RELATIVE FREQUENCY OF EACH TYPE OF RESPONSE

Number and Frequency of Masculine Responses	Total Masculine Responses	Sexual Clasp		Palpation With Pelvic Thrusts		Complete Pattern	
		Frequency	% of total responses	Frequency	% of total responses	Frequency	% of total responses
Before treatment	539	383	71	155	28	1	1
After treatment	1052	247	23	727	69	78	8

These trends in behavior and their relation to hormone treatment are further illustrated in figure 1 and table 3.

Feminine copulatory reactions. Three reactions characteristic of the receptive female rat are hopping, rapid vibration of the ears, and exhibition of lordosis when the female is mounted and palpated by a second rat. Data summarized in table 4 show that prior to the administration of male hormone the 10 castrated females were mounted by males a total of 2811 times, but none of the reactions typical of receptivity occurred in any of the 100 tests. Following androgen treatment, 4 females exhibited lordosis, 7 showed the hopping response, and 1 rat displayed the ear-wiggling reac-

TABLE 3. EFFECTS OF TESTOSTERONE PROPIONATE UPON THE RELATIVE FREQUENCY OF EACH TYPE OF MASCULINE RESPONSE

No. Tests	Mg. of Testosterone Propionate	Total Masculine Responses	Distribution of Total Responses		
			Sexual clasp	Palpation and pelvic thrusts	Complete pattern
			%	%	%
100	pre-injection	539	71	28	1
50	2–10 mg.	471	36	58	6
50	12–24 mg.	581	13	78	9

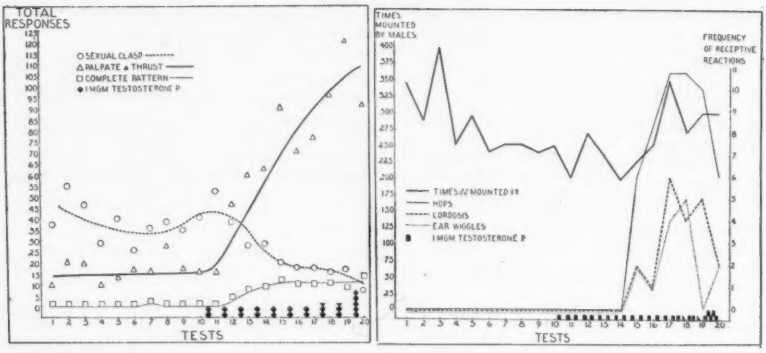

Fig. 1. Effects of androgen upon the frequency of each type of masculine copulatory response shown by 10 castrate females in 20 tests. Fig. 2. Effects of androgen upon sexually receptive behavior shown by 10 castrate females in 20 tests.

tion. The number of females fighting with the male also increased under the influence of testosterone propionate.

In table 5 the postinjection frequency of each type of estrus response is indicated. Lordosis, hopping and ear-wiggling were shown in 8, 24, and 5 tests, respectively. Although they did not appear frequently these reactions were observed a sufficient number of times so that we are sure of their occurrence.

The relationships between the mounting activity of the stimulus males, the receptive behavior of the experimental females, and the schedule of hormone injections are graphically illustrated in figure 2.

DISCUSSION

Ball reported that masculine copulatory responses of intact female rats were in-

Table 4. Effects of testosterone propionate upon the reaction of females to the copulatory attempts of males

	Times Mounted by Males	Number of Females Showing:			
		Lordosis	Hopping	Ear-wiggling	Fighting
Before androgen administration	2811	0	0	0	3
After androgen administration	2649	4	7	1	5

Table 5. Effects of testosterone propionate upon the frequency of each type of receptive behavior

Type of Female Response	Lordosis		Hopping		Ear Wiggling	
Frequency of Response	Tests in which response appeared	Total frequency	Tests in which response appeared	Total frequency	Tests in which response appeared	Total frequency
Before injection	0	0	0	0	0	0
After injection	8	20	24	44	5	14

creased by testosterone administration (8). Present findings reveal that similar results follow androgen treatment of prepuberally castrated females. Furthermore, the quantitative data herein reported support certain theoretical interpretations of the psychobiological phenomena involved.

We have previously suggested (5, 9, 10, 11) that male animals of any species differ widely in their susceptibility to sexual arousal, and that with the development of suitable tests, any individual can be assigned to a definite point upon a scale of sexual excitability. Earlier work has revealed that the sexual responsiveness of male rats may be raised by injection of testosterone propionate (10). A male's excitability is reflected in the relative frequency of incomplete copulations, complete copulations,

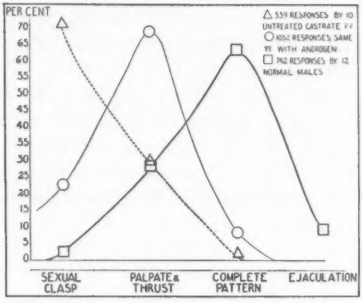

Fig. 3. Relative proportion of each type of masculine copulatory response shown by males, and by castrate females before and after androgen administration.

and ejaculations. The highly excitable male tends to execute very few palpation and thrust responses, but shows chiefly the complete pattern with frequent ejaculations. The relatively inexcitable male displays a higher proportion of incomplete copulatory reactions, fewer complete patterns, and a low number of ejaculations.

Viewed in the light of this analysis, the masculine copulatory performance of untreated castrate female rats appears qualitatively comparable to that of sexually sluggish males. A preponderance of the responses shown by females consists solely of the sexual clasp. A smaller proportion of the reactions includes palpation with pelvic thrusts, and the complete pattern is rare. Androgen administration, instead of producing a simple quantitative increase in the frequency of masculine reactions, results in a shift in the relative frequencies of the 3 types of response. And as a result of this shift the distribution of types of masculine responses becomes more nearly like that of the male.

In figure 3 the performance of the 10 castrated females before and after androgen treatment is compared with the records of 12 males observed in 52 tests in the course of an earlier experiment. The curve of masculine responses exhibited by castrated females prior to hormone administration shows a peak at the level of the sexual clasp. The mating activity of the males rises to a peak at the complete pattern level, and 8 per cent of the reactions are ejaculatory responses. Under the influence of androgen treatment the peak of the curve of female performance shifts toward that of the male curve, including a higher proportion of palpate and thrust, and complete pattern responses.

The appearance of sexually receptive reactions in the castrated females following testosterone propionate administration might be tentatively explained by assuming

that the organism transforms some fraction of the injected androgen into estrogen (12, 13). An alternative hypothesis is based upon the assumption that under certain conditions feminine sexual behavior may occur in the absence of ovarian hormones. The specific conditions involve an intense state of sexual excitement plus the repeated and forceful application of those stimuli which normally call forth receptive responses.

It may be suggested that administration of testosterone propionate markedly increases the susceptibility of the castrated female to sexual excitement. In the presence of a female in estrus the excitement of the androgen-injected rat takes overt form in the execution of masculine copulatory behavior. However, the genetically determined neuromuscular organization capable of mediating sexually receptive reactions is present in the ovariectomized female. Under ordinary conditions, feminine sexual behavior does not appear because the thresholds in the effector circuits are quite high in the absence of ovarian hormones. Nevertheless, if the castrated female, the excitability of which has been greatly increased by androgen treatment, is repeatedly mounted by a vigorous male, the neuromotor circuits for receptive behavior may be activated. Androgens strengthen the female's tendency to execute masculine behavior. They also raise her sexual excitability. When an intense state of sexual excitement exists, the application of those sensory stimuli which elicit receptive reactions in the estrus female occasionally evokes transient manifestation of the same responses on the part of the castrated female.

SUMMARY

Ten prepuberally castrated female rats were tested for masculine and feminine copulatory behavior before and after administration of testosterone propionate. Prior to androgen treatment all animals exhibited incomplete masculine mating responses. After the injection of testosterone propionate the masculine reactions of the females increased 95 per cent, and their behavior included a greater proportion of complete copulatory responses.

Although the 10 females were mounted by males 2811 times before hormone administration, no receptive behavior was exhibited. Following testosterone treatment, lordosis appeared in 4 rats, and hopping and ear-wiggling were shown by 7 and 1 females, respectively.

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COPULATORY BEHAVIOR IN PREPUBERALLY CASTRATED MALE RATS AND ITS MODIFICATION BY ESTROGEN ADMINISTRATION[1]

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IT IS frequently stated that prepuberal castration prevents the appearance of copulatory behavior in the male rat (1, 2, 3), but none of the published reports which have come to our attention includes any account of systematic attempts to elicit sexual responses in adult males castrated before puberty. Ball (4) has described the appearance of masculine mating activity in prepuberally castrated male rats treated with estrogen; but since Ball did not test for the presence of this behavior in untreated castrates the results reported cannot be attributed exclusively to the effects of the hormone.

The present study was conducted to describe and measure the masculine mating reactions of prepuberally castrated male rats before and after the administration of ovarian hormones.

METHODS

Eight male rats were castrated at 21 to 23 days of age and maintained in individual cages throughout the experiment. Between the ages of 110 and 190 days the reactions of each male to a receptive female were observed in 10 tests. These preinjection tests occurred at irregular intervals over the 2.5-month period. The final test in the control series was given after a 35-day rest, at which time the rats were 227 days of age. Four days later injections of hormone were begun. Two castrated males were given .5 mg. of progesterone[2] every day for 6 days. Five rats received 5 daily intramuscular injections of estrogen.[3] The eighth castrated male was not subjected to any hormone treatment.

Throughout the period of hormone administration and continuing at least 2 weeks after the last injection, sex tests were conducted daily with the exception of Saturdays and Sundays. Three weeks after the final injection of estrogen in oil 20-mg. pellets of crystalline estrogen were implanted in 2 males. Tests for all animals continued for another month at which time the rats were 253 days old. Each of the estrogen-treated males received a total of 20 tests after hormone administration had begun. The progesterone-injected animals were given 11 post-injection tests.

The circular testing cage was 30 inches in diameter. The test period varied in length from 10 to 30 minutes, but more than 90 per cent of the tests lasted 10 minutes. In all of the sex tests records of masculine copulatory behavior included notations as to the frequency of each element in the mating pattern of the normal male.[4] The sexual clasp

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[2] The progesterone (Proluton) was supplied by the Schering Corp., Bloomfield, N.J.

[3] The α-estradiol dipropionate (Di-Ovocylin) was furnished by Ciba Pharmaceutical Products, Inc., Summit, N.J.

[4] All tests were conducted by Miss Priscilla Rasquin whose assistance is gratefully acknowledged.

consists of mounting a second rat with the forepaws clasping the stimulus animal about the laterolumbar region. Palpation and pelvic thrusts includes the sexual clasp, but the forepaws are moved rapidly inward and downward repeatedly to 'palpate' the stimulus animal; and vigorous piston-like thrusts are executed with the hind quarters. The complete pattern involves all elements described for palpation and pelvic thrusts, and in addition, the copulating rat dismounts after a particularly forceful final pelvic thrust and runs or falls backward away from the stimulus animal, and usually licks the genitals.

RESULTS

Before the injection of any hormone, 7 of the 8 prepuberally castrated males exhibited some elements of the copulatory act. Data summarized in table 1 reveal the relative frequency of each type of response. The sexual clasp was shown 248 times.

TABLE 1. COPULATORY BEHAVIOR SHOWN BY 8 UNTREATED PREPUBERALLY CASTRATED MALE RATS IN 88 TESTS

Response	Total Frequency	Percentage of Group Showing Response	Percentage of Tests in Which Response Appeared
Sexual clasp	248	87	51
Palpation with pelvic thrusts	8	25	5

Palpation with pelvic thrusts appeared rarely; and the complete pattern did not occur.

The extracopulatory reactions of the gonadectomized animals deserve mention. Confronted with a receptive female, the castrated males gave numerous indications of intense excitement. They dashed wildly about the testing cage, often running in close circles around the female. Vigorous digging in the sawdust covering the cage floor was common. Some males lay on one side and moved all four legs in rapid running movements. When the estrous female exhibited the hopping reactions typical of receptivity the males often responded by placing their forepaws upon the female's back, and in many instances this behavior elicited lordosis. Most of the males pursued the female, crowding her roughly against the cage walls, jumping over her and often landing directly upon her back. In many tests the female's head and ears were severely bitten by the male, and males occasionally grasped the female's ear in their teeth and dragged her about the cage. This biting did not appear to constitute an attack, and females usually submitted to such treatment for several minutes without protest, although eventually fighting did develop in some cases.

The amount, intensity and duration of the sort of behavior described above appeared to be inversely related to the vigor and frequency of masculine copulatory responses. During tests in which sexual clasps were numerous the amount of such extracopulatory behavior was reduced. If masculine responses included palpation with pelvic thrusts the abortive reactions usually were not shown.

During the course of the experiment no change occurred in the behavior of the untreated castrate. The 2 males receiving injections of progesterone exhibited 6 sexual clasps in 22 tests before hormone injection and 5 such responses in 22 post-injection tests. No effects of the hormone upon behavior could be detected. Injections of estrogen exerted an appreciable effect upon the sexual reactions of castrated males, but the implantation of 20-mg. pellets of crystalline estrogen into 2 rats previously receiving estrogen in oil had no apparent results upon behavior.

The 5 males receiving estrogen in oil exhibited a total of 256 masculine copulatory responses in 88 pre-injection tests. During 134 tests following hormone administration

these animals executed 882 such reactions. Before treatment there occurred an average of 2.9 responses per test, and estrogen increased this figure to 6.6 per test. Test-to-test records for this group of males are shown in figure 1.

Data represented in figure 1 show that during the period of estrogen administration the frequency of the sexual clasp decreased, while palpation with pelvic thrusts increased. Immediately following the injections the sexual clasp returned to approximately normal frequency, palpation with pelvic thrusts continued to increase and the complete pattern appeared. Tables 2 and 3 further illustrate the fact that estrogen increased the frequency of palpation with pelvic thrusts and of the complete pattern.

In the normal male, palpation with pelvic thrusts is usually labelled an 'attempt' and constitutes an unsuccessful or incomplete copulation. The complete pattern is termed a 'copulation' since it nearly always indicates the occurrence of intromission.

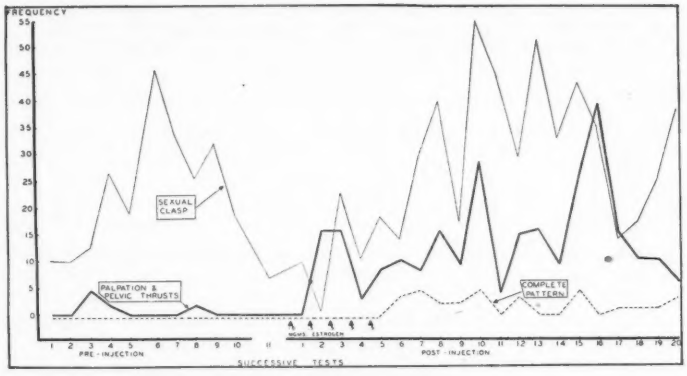

Fig. 1. TOTAL FREQUENCY of each type of masculine copulatory response in pre- and postinjection tests.

In the present study the estrogenized castrate which showed the complete pattern exhibited this response a total of 28 times in 11 tests. Repeated examination indicated that penile erection never occurred.

DISCUSSION

The failure of earlier workers to observe copulatory responses in prepuberally castrated male rats is probably due to inadequate testing techniques. In fact, the only report in which any sex tests are specifically mentioned is that of Shapiro (1) who merely states that when prepuberally castrated males were placed with females 9 weeks after gonadectomy 'they showed no sexual behavior.' Apparently only 1 test was given. Copulatory reactions have been observed in prepuberally castrated male guinea pigs (5, 6, 7), and pigeons (8), in capons (9, 10, 11), and in pigeons in which gonads are congenitally absent (12). Present findings with the rat are thus in agreement with results obtained on other vertebrate species.

Practically all observers have noted that the sexual reactions of males castrated before puberty are less vigorous and complete than the copulatory performance of the normal animal. These results strongly indicate a threshold phenomenon. It may be suggested that the neuromotor circuits mediating the masculine mating pattern are organized well in advance of puberty, and that testicular hormones lower the

threshold in these circuits. The hormonal secretions of the rat testis show a marked and abrupt increase at 35 to 40 days of age (3), and copulatory behavior makes its first appearance at the same time (13). This concomitance represents the activation of a behavior pattern by hormonal reduction of neuromotor thresholds. That the nervous and muscular organization is complete before the hormone is available is indicated by the ease with which the behavior may be elicited in prepuberal males under the influence of injected androgen (rats 13, chicks 14).

We are inclined to doubt the validity of the statement that 'hormones from the

TABLE 2. EFFECTS OF ESTROGEN UPON EACH TYPE OF MASCULINE COPULATORY RESPONSE IN 5 CASTRATED MALES

	Sexual Claps			Palpate and Thrust			Complete Pattern		
	Cases showing response	Tests in which response appeared %	Average frequency per positive test	Cases showing response	Tests in which response appeared %	Average frequency per positive test	Cases showing response	Tests in which response appeared %	Average frequency per positive test
50 tests before hormone administration	5	80	5.84	2	8	2.00	0	0	0
100 tests after hormone administration	5	79	5.05	3	30	8.93	1	11	2.55

TABLE 3. RELATIVE FREQUENCY OF EACH TYPE OF COPULATORY RESPONSE BEFORE AND AFTER INJECTION OF ESTROGEN

	Total Response	Sexual Clasp		Palpate and Thrust		Complete Pattern	
		Frequency	% of Total	Frequency	% of Total	Frequency	% of Total
50 Pre-injection tests	242	234	97	8	3	0	0
100 Post-injection tests	802	505	61	269	33	28	6

gonads are the *sine qua non* for the organization of sexual behavior in young vertebrates' (2). Sexual arousal is best interpreted as a product of two independent variables, *a*), the responsiveness of the male, and *b*), the excitatory value of the stimulus situation. There are marked individual differences in the ease with which different males of any species may be brought to the point of copulation. Further, each individual's excitability is a joint product of nervous and hormonal influences. At least a part of the male's susceptibility to sexual arousal is dependent upon genetically organized neural mechanisms, and gonadal hormones greatly increase excitability by lowering the thresholds within these mechanisms. In some individuals, however, the thresholds may be passed even in the absence of testicular secretions, providing the excitatory value of the stimulus situation is sufficiently intense (15). All observers agree that the elicitation of sexual reactions in prepuberally castrated males involves the employment of very strong stimulation (16).

Sexual excitement is obviously not an all-or-none phenomenon. The execution of the complete copulatory pattern does not demand quite so high a degree of excitement as does the occurrence of ejaculation. Even lesser degrees of sexual arousal are associated with the display of incomplete copulatory attempts. When prepuberal castrates are confronted with a receptive female the degree of arousal may be sub-

maximal. The prepuberally castrated males described in the present report gave unquestionable evidence of sexual arousal. In some cases the excitement was sufficiently intense to result in overt mating reactions, but in the absence of gonadal hormones it did not rise to the level necessary for the occurrence of the complete copulatory pattern.

Stone (17) treated male rats castrated in adulthood with theelin and obtained results which he regarded as 'equivocal.' Sexual activity was increased in some cases but not in others.[5] Ball (19) reported restoration of copulatory activity in castrated adult male rats following estrogen administration. Both of these studies dealt with animals in which testicular hormones had been secreted postpuberally for several months prior to castration. Ball's study (4) of prepuberally castrated male rats given estrogen revealed the occurrence of sporadic copulatory attempts; but since animals were not tested before hormone treatment the data, as Ball wrote, did 'not show whether or not estrogen is necessary to produce male behavior.'

Our results indicate that although untreated prepuberal castrates are capable of some copulatory behavior, the administration of estrogen increases sexual excitability. As a result of heightened excitability there is an increased frequency of palpation with pelvic thrusts and the complete copulatory pattern appears. It is suggested that estrogen tends to lower slightly the thresholds in neuromotor circuits mediating masculine copulatory behavior in the male rat.

SUMMARY

Male rats castrated at 21 days of age and tested with receptive females at 100 days showed sexual excitement and some copulatory activity. Mating attempts consisted chiefly of mounting and clasping the female, with the occasional occurrence of palpation and pelvic thrusts.

Administration of estrogen increased the frequency of responses involving palpation and pelvic thrusts, and resulted in the appearance of the complete pattern typical of the normal copulating male. The pattern of ejaculation was not observed.

It is suggested that in the prepuberal castrate a low but definite degree of sexual arousal is possible. Estrogen raises excitability appreciably, permitting the occurrence of copulatory reactions more nearly like those of the intact male.

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[5] This is not surprising since even androgen administration sometimes fails to produce sexual behavior in male rats (13, 18).

CARDIOTOXIC GOITER—A DISTINCT ENTITY

A PRELIMINARY REPORT[1]

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IT IS GENERALLY TAUGHT that the cardiac manifestations of thyrotoxicosis occur as a part of and incidental to accelerated tissue metabolism resulting from excessive thyroid secretion. Several cardiologists of repute maintain that, unless there is an underlying pathologic lesion, there is no such thing as a 'goiter heart.' Their views are supported by the observation that no characteristic pathologic alteration is demonstrable in microscopic sections of the myocardium of thyrocardiac patients. Further, it is generally held that the thyroid hormone, thyroxine, is the active principle of the thyroid secretion. Thus, thrytoxicosis, whether associated with a diffuse or with a nodular goiter, results simply from hyperthyroidism.

Adherents to the concept of thyroid hypersecretion employ basal metabolic rate determinations for the purpose of *a*), diagnosing hyperthyroidism, and *b*), estimating the degree of hyperthyroidism.

The foregoing hypothesis is attractive because of its simplicity. Unfortunately, upon careful consideration, not even the majority of goiters producing thyrotoxic manifestations can be fitted into this category. Notable among such exceptions are 'burned out' diffuse hyperplastic goiters, nodular or degenerated colloid goiters of long duration, and old degenerated fetal adenomas. In all types of goiters of many year's duration, cardiac disturbances eventually become the most dominant feature of the thyrotoxic state, while significantly elevated metabolic rates become the exception rather than the rule. Various authors have written about this type of goiter under such paradoxical titles as 'chronic hyperthyroidism with normal metabolic rate' and 'apathetic hyperthyroidism' (1–4). Obviously, in the absence of elevated metabolism, the thyrotoxic symptoms cannot be due to excessive thyroxine liberation. Inasmuch as thyroidectomy alleviates the thyrotoxic symptoms the question of an abnormal thyroid secretion becomes a subject for investigation.

The colloid of all toxic goiters, both diffuse and nodular, is abnormal. This statement is based on studies made during the past several years in this laboratory which have demonstrated *a*), changes in the staining properties of the colloid (one of us—A. E. H.—first began this phase of the investigation in 1899), *b*), chemical alterations in the thyroglobulin obtained from goiters removed surgically, and *c*), the presence in goiter extracts of an organic iodine-containing substance other than thyroxine which, when fed to thyroidectomized rats, causes an increased heart rate without a corresponding increase in the metabolic rate.

Alteration in staining properties. The colloid of the normal thyroid stains uniformly pink with a faintly bluish tinge with hematoxylin-eosin stain. With Mallory's

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[1] This communication is presented as a preliminary report, a result of the present National Emergency which for the time being has interrupted this investigation. It is hoped that detailed reports will appear in the proper basic science journals at a later date. C.R.S.

aniline blue stain the colloid takes a uniform, deep blue stain. Changes in the staining characteristics of the colloid are best demonstrated in old nodular goiters that have finally become toxic. The most striking change is that the colloid which is normally acidophilic with hematoxylin-eosin stain becomes basophilic. These follicles take a dark, pinkish-blue stain. As a rule the change does not involve all of the follicles but only the colloid of an individual acinus which may be surrounded by acini containing acidophilic colloid. This precludes the possibility of an artifact. Rarely, all of the acini show basophilic changes but in varying degree. Occasionally there is evidence of degeneration of the acinar epithelium with the colloid staining poorly, or not at all.

When stained with Mallory's aniline blue dye the change in staining properties

TABLE 1. DISTRIBUTION OF ORGANIC IODINE IN CARDIOTOXIC GOITER

Case No.	Type of Goiter	Age of Patient	Duration	Heart Symptoms	% Total Organic Iodine	% Thyroxine Iodine[1]	% of Total as Thyroxine
		yr.					
	Normal thyroid	23			0.360	0.150	41
	Normal thyroid	27			0.320	0.120	37
21471	Nodular colloid	57	25 yr.	2 yr.	0.093	0.027	29
21747	Toxic diffuse hyperplastic	56	?	1 yr.	0.061	0.006	10
21736	Nodular toxic colloid	58	12 yr.	2 yr.	0.150	0.050	33
21880	Toxic exophthalmic	19	1 mo.	10 mo.	0.170	0.033	19
21722	Diffuse hyperplastic toxic	28	4 mo.	4 mo.	0.150	0.029	19
21874	Toxic nodular colloid	67	25 yr.	1 yr.	0.120	0.010	8
21730	Toxic exophthalmic	32	13 yr.	4 yr.	0.310	0.070	22
21914	Diffuse hyperplastic toxic	46	2 mo.	2 yr.	0.220	0.035	16
21836	Recurrent exophthalmic. (2nd stage)	47	20 yr.	1 yr.	0.270	0.030	11
21740	Diffuse hyperplastic	40	6 yr.	6 yr.	0.280	0.019	7

[1] Method of Harington and Randall, *Quart. J. Pharm. & Pharmacol.* 2: 501. 1929.

is even more striking. Here the normally basophilic colloid stains, instead of deep blue, a brilliant orange. As with the hematoxylin-eosin stain, it is only rarely that the colloid of all the acini stain orange with Mallory's dye. More commonly, the colloid of individual follicles stain orange with that of the surrounding acini taking varying shades of blue. Occasionally one sees an intermingling of blue and orange in the same follicle.

The same changes occur also in the burned out diffuse goiters of long duration; however, in these goiters one encounters more frequently degeneration of the epithelium, sometimes with desquamation of the acinar cells into a colloid that stains faintly or is absent.

BIOCHEMICAL AND BIOLOGICAL ASSAY

Several prerequisites are imperative in a study of this nature. First, in preparing an extract from human goiters, measures to prevent autolysis of the fresh specimen must be taken. Next, the method of preparation should eliminate the possibility of denaturation of the thyroid protein. Lastly, the extract must be stable and uniform so that an aliquot is representative of the entire gland.

As soon as the specimens were removed surgically several small areas were taken for microscopic study and the remainder of the gland was immediately frozen solid in the freezing compartment of a refrigerator. In the frozen state the gland was then shaved into thin slices which were thrice extracted at ice-box temperature with

0.85 per cent sodium chloride. The three saline extracts were combined and dehydration accomplished by means of the lyophile technic of rapid freezing and removal of water vapor under high vacuum (5). The finished product was a uniform crystalline, porous mass readily soluble in water.

Chemical analysis. Determinations of total iodine, inorganic iodine and thyroxine iodine were made on 26 goiters and 2 normal thyroid glands. Details of the analysis of 10 of the goiters appear in table 1 and can be summarized as follows.

Thyroglobulin obtained from normal thyroids in the manner described above contains from 0.2 to 0.4 per cent total iodine. Of this, 7 per cent is present in the inorganic state while the remaining 93 per cent is in the organic form. Approximately one-half of the organic iodine (less with some methods of assay) exists in the thyroxine molecule. The remaining 50 per cent is bound up with iodoproteins other than thyroxine, chiefly di-iodotyrosine. The latter is thought to be a precursor to thyroxine, but in itself does not possess metabolism-stimulating properties.

TABLE 2. EFFECT OF GOITER EXTRACT ON METABOLISM AND HEART RATE OF THYROIDECTOMIZED RATS

Rat No.	mg. Oxygen per kg. per hr.			Heart Rate		
	Before	After	% Change	Before	After	% Change
1	1350	980	−27	160	300	+88
2	1230	1095	−11	180	300	+67
3	1500	1600	+ 7	156	300	+92
4	1108	1000	−10	150	277	+85
5	1082	1090	0	144	276	+92
6	960	1030	+ 7	180	300	+67
7	1050	1000	− 5	160	300	+88
8	1008	1095	+ 9	220	330	+50
9	1100	1110	+ 1	176	360	+84
10	880	1080	+23	240	360	+50

Goiter Extract from *Patient 21874* (table 1). Total 3-day intake of goiter extract, 1500 μg. per 100 gm. of rat; total 3-day thyroxine-iodine intake, 15 μg. per 100 gm. of rat; total 3-day organic iodine intake, 180 μg. per 100 gm. of rat.

From the data in table 1 it is apparent that the colloid from goiters differs from that of the normal thyroid in that there is *a*), a decrease in total iodine, *b*) a decrease in thyroxine iodine, and *c*) an increase in the nonthyroxine organic iodine fraction.

The above was found to be the case after several weeks of pre-operative therapy with Lugol's solution. This indicates that the goitrous gland is deficient in two respects. It cannot convert organic iodine into thyroxine in normal amounts. It is incapable of storing normal amounts of iodine, which confirms the report of Cavett et al. (6).

As subsequent studies of these goiter extracts will suggest, it appears that the toxic substance or substances liberated by a dysfunctioning thyroid gland arise in the nonthyroxine organic iodine-containing fraction. On this basis, thyrotoxicosis results, then, not from excessive thyroxine manufacture and liberation, but from the liberation of an altered secretion containing intermediary precursor molecules having toxic properties.

On the basis of chemical analysis of extracts from all types of goiter we conclude, as did Cavett, Rice and McClendon (6), that, from the standpoint of the colloid, there is but one type of goiter, namely, a dysfunctioning thyroid gland which is incapable of producing normal thyroglobulin.

Effect of goiter extracts on rate of oxygen consumption and heart rate of thyroid-

ectomized rats. The removal of all of the thyroid gland from 60-day-old rats results in retarded growth. At the end of one month there is a decrease in the rate of oxygen consumption of about 40 per cent and a decrease in the heart rate from the normal of 300 beats per minute to about 150 beats. Heart rates in these studies were accurately determined by electrocardiograph tracings before and after extract feeding, with the animals under light nembutal narcosis. The rate of oxygen consumption was determined in a closed-system metabolism chamber provided with two soda lime (Dioxorb) trays for the absorption of expired carbon dioxide (7). Six weeks were allowed to elapse after thyroidectomy before oxygen consumption determinations were begun. Food was withheld 24 hours before each test. Tests were repeated every 4th day until the animals became accustomed to the metabolism chamber and until two consecutive determinations checked within 10 per cent. As an additional check on the accuracy of the oxygen consumption determinations, a normal rat was included with each series of thyroidectomized animals.

When a thyroidectomized rat is fed thyroxine or commercial thyroid extract it is possible to administer a dosage which will elevate the rate of oxygen consumption to the normal level, with an associated increase in heart rate to a rate of normal 300. Meyer (8), using this procedure for bio-assay, found that, following hydrolysis with acids and alkali, certain fractions of commercial thyroid extract did not produce a comparable rise in oxygen consumption and heart rate when administered to a-thyroid rats. This suggested that metabolic increases and increased heart rate may not be due solely to thyroxine. This same investigator has more recently (9) fed bits of fresh goiters obtained from surgical patients and obtained results that were inconclusive; however in some instances he observed increased heart rates without a corresponding rise in the rate of oxygen consumption.

The practice of feeding bits of fresh thyroid gland in such experiments is open to criticism on the basis that one must make the unwarranted assumption of uniformity of structure and colloid content which obviously does not obtain in a goiter. It was for this reason that, early in 1941, we took special care to secure a uniform extract which was representative of the entire goiter.

Five of the goiter extracts, results with which are reported in table 1, have been fed to a series of 10 thyroidectomized rats in which the rates of oxygen consumption averaged around 40 per cent below normal and the heart rates averaged around 150 beats per minute. The extracts were administered in solution by stomach tube in amounts determined by the thyroxine content of the individual goiters. Five micrograms of thyroxine iodine per 100 gm. of rat was the standard daily dose. It should be pointed out that, on this basis, the total dose of extract varied inversely with the thyroxine content. Thus, the lower the thyroxine content, the larger the total intake of extract.

As soon as the rate of oxygen consumption became stabilized, determinations of the heart rate were made and goiter extract was then fed for 3 consecutive days. The rate of oxygen consumption and of the heart were determined again on the 5th day. Typical results in one such experiment are shown in table 2. Without significant change in the oxygen consumption rates, the heart rates were increased from 50 to 110 per cent in all test animals. It is evident that extracts from toxic goiters contain a cardiac-stimulating principle which, by definition is not thyroxine, since it does not induce metabolic stimulation. The liberation of this principle in the altered secretion of a toxic goiter could adequately explain the cardiac irritability of clinical 'chronic hyperthyroidism' with normal metabolic rate.

Many problems remain for investigation. In the first place a larger number of

experiments must be conducted to establish unequivocally the presence of a specific, non-thyroxine cardio-stimulant in toxic goiter extracts. We have not as yet extended this type of biological assay to earlier forms of diffuse and nodular goiter, nor have we attempted to correlate the clinical toxicity of the goiter with the relative potency of the goiter extract as a cardiostimulant. Purification of the non-thyroxine organic iodine fraction of toxic goiter extracts should be attempted. Toxicity studies on normal rats using metabolic rate determinations, heart activity observations with the electrocardiograph, together with histologic studies of the heart from normal animals which have received large doses of thyroxine-free extract, remain for investigation.

CONCLUSIONS

The present-day concept that the clinical manifestations of toxic goiter are due to an excessive production of thyroxine fails to explain chronic hyperthyroidism with a normal metabolic rate. We have obtained three types of evidence which indicate that the colloid of goiters is abnormal.

1. In toxic goiter there is an associated change in the staining properties of the colloid. In some, the colloid is thin, staining poorly or not at all; in others the staining reaction of the colloid of certain follicles changes from acidophilic to basophilic. With Mallory's stain these areas stain orange instead of the usual blue.

2. Chemical analysis of thyroglobulin from toxic goiters after iodine therapy reveals alterations in the distribution of organic iodine; thyroxine iodine is diminished while the nonthyroxine organic fraction is appreciably increased. No goiter was found to contain normal amounts of total iodine.

3. Extracts from toxic goiters contain a principle which stimulates the heart rate of thyroidectomized rats without an associated increase in the rate of oxygen consumption.

It is concluded that the clinical picture of thyrotoxicosis may be better explained on the basis of a perverted thyroid secretion instead of on the basis of an excess of normal secretion.

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NOTES AND COMMENTS

PROLACTIN AND HEALING OF EXPERIMENTAL PEPTIC ULCER

Extracts prepared from the urine of pregnant and nonpregnant women have been found to have prophylactic and therapeutic effects on the experimental peptic ulcer of the jejunum in dogs.[1,2] The incidence of ulcer is very greatly reduced by treating the dogs with these urine extracts. In treated animals, in which ulcers have occurred, there are found definite histological evidences of healing, consisting of fibroblastic proliferation and epithelialization of the ulcer area. Such signs of healing are very seldom found in animals not treated with either normal or pregnancy urine extracts.

Attempts to identify the organ responsible for the elaboration of the active principle have so far yielded negative results.[3] The only finding made to date on this point is the almost total absence of the ulcer-protecting principle in the urine of patients with symptoms of active peptic ulcer of the duodenum.[1,2] Recently Schooley, Plummer, Riddle and Bates[4] reported that extracts of anterior pituitary, which showed very high prolactin activity, are capable of increasing intestinal weight and villus length in normal as well as hypophysectomized pigeons. They suggest that this splanchnomegaly is similar to that produced more slowly in mammals by the injection of pituitary extracts.[5] The excretion of prolactin in urine from men, pregnant and nonpregnant women and infants has been reported.[6,7] We, therefore, considered the possibility that the tissue proliferation at the site of the ulcer in urine-treated animals might be due to anterior pituitary substance with high prolactin activity. Extracts of urine from pregnant and nonpregnant women, which are known to be beneficial in healing of the experimental ulcer, were tested for prolactin. Pigeons weighing 295 to 380 gm. were used. Two groups of 12 birds were each given daily 6 and 12 mg., respectively, of the urine extracts (6 mg. per cc. in 0.5% phenol). These dosage levels are considerably higher than the 1 mg. per day found effective in treating the experimental ulcer. The injections were made once daily for 4 days in the breast muscle just adjacent to the crop of the pigeon. On the 5th day the crops were examined for increase in weight (as compared with 12 untreated control pigeons) and for proliferation of the crop glands, as suggested by McShan and Turner.[8]

None of the treated birds showed a gain in crop weight. Unmistakable evidences of crop proliferation were found in one bird of the control series and none in the urine-treated series. Therefore, we must conclude that urine extracts which can cause fibroblastic proliferation and epithelialization of the experimental ulcer do not do so because of prolactin. While this study excludes prolactin as the active agency in urine, it does not rule out the action of other anterior pituitary substances which may be excreted in the urine. Furthermore, this study does not exclude the possibility that potent preparations of prolactin (which these

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[1] Sandweiss, D. J., H. C. Saltzstein and A. A. Farbman: *Am. J. Digest. Dis. & Nutrition* 5: 24. 1938.

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[4] Schooley, J. P., O. Riddle and R. W. Bates: *Am. J. Anat.* 69: 123. 1941.

[5] Reichert, F. L., M. E. Simpson, P. G. Cornish and H. M. Evans: *Mem. U. California* 2: 413. 1933.

[6] Lyons, W. R., and E. Page: *Proc. Soc. Exper. Biol. & Med.* 32: 1049. 1935.

[7] Lyons, W. R.: *Proc. Soc. Exper. Biol. & Med.* 37: 207. 1937.

[8] McShan, W. H., and C. W. Turner: *Proc. Soc. Exper. Biol. & Med.* 34: 50. 1937.

urine extracts were not) may likewise confer some beneficial effect on the experimental ulcer.

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INDUCTION OF MALE COPULATORY BEHAVIOR IN A HEN FOLLOWING ADMINISTRATION OF MALE HORMONE

IT IS WELL-KNOWN that the administration of male hormone substance to hens (*Gallus domesticus*) induces masculinization of the secondary sex characters. Hamilton[1] has shown that these somatic changes are accompanied by certain behavioral responses typical of the rooster. Following injection or implantation of male sex hormone hens will crow, display cocklike fighting, and exhibit male courtship activity, *i.e.*, 'waltzing.' However, the display of masculine copulatory behavior by these birds has never been observed. The absence of this element of behavior has long puzzled investigators, in view of the ease with which other aspects of masculinization are induced. The present report deals with a single hen in which masculine copulatory behavior was observed following androgen treatment.

On July 2, 1941 each of two White Leghorn pullets, exactly 5 months old, received a subcutaneous implantation of a pellet of testosterone propionate weighing approximately 41 mg.* Egg-laying had not yet begun. Regular tests for masculine behavior were conducted for more than a month, but no behavioral changes occurred, and observations were discontinued. On November 17, 4.5 months after the date of pellet implantation, an observer saw one of these birds, *female 21*, treading a hen. Palpation revealed that approximately one-third of the pellet was still intact. There was no trace of the pellet in the second bird, which by this time had completely reverted to hen-like appearance and behavior.

Female 21 was tested at regular intervals with a squatting hen and with a sexually-active male. Numerous treadings were observed in which the female exhibited typical masculine behavior. 'Waltzing' was only occasionally seen and was never very vigorous. Crowing was heard on one occasion only, when after she was treaded by a male, *female 21* emitted three distinct crows. The masculine copulatory response continued to be exhibited by this bird until late December, although the pellet could not be palpated after December 14. On Jan. 1, 1942, *female 21* laid its first egg, trap-nesting precluding the possibility of error as to the identity of the layer. Other eggs were subsequently laid, one of which was incubated and proved to be fertile. The hen was killed on March 11 and examination re-confirmed its sex.

Attempts are being made to repeat the results reported here using a larger group of birds.

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[1] HAMILTON, J. B.: *Endocrinology* 23: 53. 1938.

* Crystalline testosterone propionate (Perandren), supplied by Ciba Pharmaceutical Products, Inc., Summit, N. J.

ASSOCIATION NOTICE

ANNOUNCEMENT OF THE ANNUAL MEETING

The meeting herein announced will be held contingent upon authorization by the Office of Defense Transportation

The TWENTY-SEVENTH ANNUAL MEETING of The Association for the Study of Internal Secretions will be held in Cleveland, Ohio, on Monday and Tuesday, April 5 and 6, 1943. The Hotel Cleveland will be the headquarters for registration and for the scientific and business sessions. Room reservations should be made early with the Hotel Cleveland.

The Chairman of the Local Committee is Dr. E. Perry McCullagh, Cleveland Clinic, Cleveland, Ohio.

PRESENTATION OF PAPERS

1. The title of the paper and four copies of a comprehensive abstract must reach the President, Dr. E. Kost Shelton, 921 Westwood Boulevard, Los Angeles, California, not later than February 10, 1943.

Abstracts submitted should be in proper form for printing in ENDOCRINOLOGY. Not more than the first 200 words can be included in the printed abstract.

2. Non-members who wish to present papers must have their titles and abstracts introduced by members.

3. The abstracts of papers will be considered by the Program Committee and the final program announced about March 10, 1943.

4. Papers will be limited to ten minutes for presentation. A ten-minute presentation is designed for condensed discussion of new investigations, either in laboratory or clinical phases of endocrinology. Previous publication or presentation before other societies of national membership may be cause for omitting a paper from the program.

5. Papers presented at the Annual Meeting may be submitted for publication to the Editor of ENDOCRINOLOGY or the JOURNAL OF CLINICAL ENDOCRINOLOGY. Such submission is invited. Acceptance of a paper for the program does not necessarily mean its acceptance for publication.

You are invited to bring this announcement to the attention of your colleagues and associates who have endocrine research in progress.

NOMINATION OF OFFICERS

The By-Laws of the Association provide that nominations for all elective offices shall be made by a Nominating Committee and forwarded to the Secretary at least sixty days before the annual meeting. The Nominating Committee for the current year, appointed by the President and accepted by the Council, is as follows:

Dr. E. Perry McCullagh, *Chairman*, Cleveland Clinic, Cleveland, Ohio.
Dr. C. N. H. Long, Yale University School of Medicine, New Haven, Connecticut.
Dr. Warren O Nelson, Department of Anatomy, Wayne University, Detroit, Michigan.

The By-Laws also provide (Article V, Section 2) that "Any member of the Association may submit nominations to the Nominating Committee for its consideration." Nominations may be sent to the Chairman, Dr. E. Perry McCullagh, before February 1, 1943.

The terms of the following officers expire at the time of the Annual Meeting in Cleveland in 1943:

President-Elect	Carl R. Moore
Vice-President	John C. Burch
Secretary-Treasurer	Henry H. Turner

Council Members	*Publication Board*
P. E. Smith	E. C. Hamblen
M. A. Goldzieher	Carl R. Moore
George W. Thorn	

THE E. R. SQUIBB & SONS AWARD

The E. R. Squibb & Sons Award of $1,000.00 was established in 1939, and was given first in 1940 to Dr. George W. Corner, in 1941 to Dr. Philip E. Smith, and in 1942 to Dr. Fred C. Koch. A special Committee of five members of the Association chooses an investigator or investigators in the United States or Canada for one of the best contributions to endocrinology.

THE CIBA AWARD

The Ciba Award to recognize the meritorious accomplishment of an investigator not more than 35 years of age in the field of endocrinology was established last year but no recipient was selected due to lack of time. The work cited may be either in the field of pre-clinical or clinical endocrinology. The Award is for $1200.00. If the recipient should choose to use the Award toward further study in a laboratory other than that in which he is at present working, the Award will be increased to $1800.00. The option is left entirely to the recipient.

Each member has the privilege of making one nomination for each Award. A nomination should be accompanied by a statement of the importance of the nominee's contributions in endocrinology and by a bibliography of the nominee's most important publications, and reprints if possible. Five copies should be sent to the Secretary, Dr. Henry H. Turner, 1200 North Walker Street, Oklahoma City, Oklahoma, not later than February 1, 1943.

E. Kost Shelton, *President*
Henry H. Turner, *Secretary*

PROCEEDINGS OF THE TWENTY-SIXTH ANNUAL MEETING

THE TWENTY-SIXTH ANNUAL MEETING of the Association for the Study of Internal Secretions was held in Atlantic City, New Jersey, at the Hotel Traymore on June 8th and 9th, 1942. A total of 328 people registered, 198 of whom were members.

The meetings consisted of a general session the morning of the first day, with a symposium on the relation of endocrines to skeletal growth in the afternoon; on the second day simultaneous sessions were held both morning and afternoon in the Belvedere and Stratosphere rooms of the Hotel Traymore. Seventy-six papers were presented, fourteen of which were read by title. Abstracts of the papers were pub-

lished in the June issue of ENDOCRINOLOGY which was released to the public at the meeting.

The annual dinner was held on Monday evening, at which a formal presentation of the twenty-fifth anniversary number of ENDOCRINOLOGY, volume 30, number 6, was made. The Presidential Address, 'A Picture Album of Tumors in Experimental Animals following Estrogenic Treatment' was delivered by Dr. Edgar Allen.

The third E. R. Squibb & Sons Award was made to Dr. Fred C. Koch for his work on the androgens.

The Ciba Award was not made for 1942.

COUNCIL MEETINGS AND BUSINESS MEETING

The first session of the Council was held at 6:30 P.M., June 7, 1942 in the Belvedere Room of the Hotel Traymore. Reports of the Nominating Committee, the Awards Committee and the Publication Board were made and accepted. The second meeting of the Council was held at 7:30 A.M., June 9th. The resignation of Dr. M. O. Lee as Managing Editor of ENDOCRINOLOGY and the JOURNAL OF CLINICAL ENDOCRINOLOGY was accepted. The Council appointed Dr. R. G. Hoskins as Acting Managing Editor. Dr. Hoskins and the officers for 1942–43 were instructed to act as a committee to survey the conduction of the Association and its journals and to bring in to the Council any recommendations for changes that it should deem advisable. The Council reconvened at 12:30 P.M., June 9th. Reports of the Committee on Assays and the Committee for the Twenty-fifth Anniversary Number of ENDOCRINOLOGY were accepted.

The following persons were elected by the Council to active membership in the Association:

ABARBANEL, ABRAHAM R., M.D., *Washington, D. C.*
ALLEN, FREDERICK M., M.D., *New York, N. Y.*
ARNOW, L. EARLE, M.D., *Glenolden, Penn.*
ANSBACHER, S., D.Sc., *Richmond Hill, N. Y.*
BAKER, BURTON LOWELL, Ph.D., *New York, N. Y.*
BISCHOFF, FRITZ E., Ph.D., *Santa Barbara, Calif.*
BLANCHARD, ERNEST W., Ph.D., *New York, N. Y.*
BRANDALEONE, HAROLD, M.D., *New York, N. Y.*
BROUGHER, JOHN C., M.D. (reinst.), *Vancouver, Wash.*
BRUES, AUSTIN M., M.D., *Boston, Mass.*
CHEN, K. K., M.D., *Indianapolis, Ind.*
COLTON, NATHAN H., M.D., *Philadelphia, Penn.*
DORFMAN, RALPH I., Ph.D., *Cleveland, Ohio*
ENGEL, FRANK LIBMAN, M.D., *New Haven, Conn.*
EVERETT, JOHN W., Ph.D., *Durham, N. C.*
FARQUHARSON, RAY F., M.B., *Toronto, Ontario, Canada*
FIGGE, FRANK H. J., Ph.D., *Baltimore, Md.*
FIRST, ARTHUR, M.D., *Philadelphia, Penn.*
FLEISCHMANN, WALTER, M.D., *Baltimore, Md.*
FRAZER, MARY MARGARET, M.D., *Detroit, Mich.*
GAEBLER, OLIVER HENRY, M.D., *Detroit, Mich.*
GAY, MICHEL, M.D., *Los Angeles, Calif.*
GILLMAN, JOSEPH, M.D., *Johannesburg, South Africa*
GRANGER, GORDON A., M.D., *Washington, D. C.*
GREENE, JAMES A., M.D., *Iowa City, Iowa*
GURIN, SAMUEL, Ph.D., *Philadelphia, Penn.*
GUSMAN, HARRY A., M.D., *Cleveland, Ohio*
HATERIUS, HANS O., Ph.D., *Detroit, Mich.*
HECHTER, OSCAR, B.S., *Los Angeles, Calif.*
HETHERINGTON, ALBERT W., JR., Ph.D., *Chicago, Ill.*
HEMING, ARTHUR EDWARD, Ph.D., *Sao Paulo, Brazil*
HIRSCHMANN, HANS, M.D., Ph.D., *Cleveland, Ohio*
HOOKER, CHARLES W., Ph.D., *New Haven, Conn.*
HORNING, ERIC, D.Sc., *London, England*
HOSTER, HERMAN A., M.D., *Columbus, Ohio*
INGLE, DWIGHT J., Ph.D., *Kalamazoo, Mich.*
JAILER, JOSEPH WILLIAM, Ph.D., *New York, N. Y.*
JANES, RALPH G., Ph.D., *Detroit, Mich.*
JEFFERS, KATHARINE R., Ph.D., *Durham, N. C.*
JONES, HERMAN D., Ph.D., *Bristol, Tenn.*
KEATING, F. RAYMOND, JR., M.D., *Rochester, Minn.*
KOCHAKIAN, CHARLES DANIEL, Ph.D., *Rochester, N. Y.*
KOTZ, J., M.D., *Washington, D. C.*
LOEB, ROBERT, M.D., *New York, N. Y.*
MAYER, DENNIS T., Ph.D., *Columbia, Mo.*
MELLA, CHARLES A., JR., M.D., *Nashville, Tenn.*
MENKIN, VALY, M.D., *Boston, Mass.*
NADLER, SAMUEL B., M.D., Ph.D., *New Orleans, La.*
NICHOLAS, J. S., Ph.D., *New Haven, Conn.*
PAPAGEORGE, EVANGELINE, Ph.D., *Emory University, Atlanta, Ga.*
PERLOFF, WILLIAM H., M.D., *Philadelphia, Penn.*
PFEIFFER, CARROLL A., Ph.D., *New Haven, Conn.*
PHILLIPS, COLGATE, M.D., *Bronxville, N. Y.*
PIRK, LEO A., Ph.D., *Nutley, N. J.*
RHEUBY, VIRGINIA GOULD, M.D., *Philadelphia, Penn.*

ROOT, HOWARD, F., M.D., *Boston, Mass.*
RUSSELL, JANE A., Ph.D., *New Haven, Conn.*
SEIDLIN, SAMUEL M., M.D., *New York, N. Y.*
TAYLOR, HOWARD CANNING, JR., M.D., *New York, N. Y.*
TEPPERMAN, JAY, M.D., *New Haven, Conn.*
THANNHAUSER, SIEGFRIED, M.D., Ph.D., *Boston, Mass.*
TORREY, EUGENE W., M.D. (reinst.), *Perry Point, Md.*
VARGAS, LUIS, M.D., *Santiago, Chile.*
WEBER, FREDERICK C., Jr., M.D., *Greenwich, Conn.*
WEICHERT, CHARLES K., Ph.D., *Cincinnati, Ohio*
WHITE, PRISCILLA, M.D., *Boston, Mass.*
WILKINS, LAWSON, M.D., *Baltimore, Md.*
WILLIAMS, W. LANE, Ph.D., *Rochester, N. Y.*
WILLIER, B. H., Ph.D., *Baltimore, Md.*
WINTERSTEINER, OSKAR PAUL, Ph.D., *New Brunswick, N. J.*

Dr. J. P. Pratt was appointed by the President to serve the unexpired term of Dr. E. A. Doisy, resigned, on the Awards Committee. The Nominating Committee for 1942–43 was appointed by the President-elect, Dr. E. K. Shelton, the members of which were Dr. E. Perry McCullagh, Chairman, Dr. C. N. H. Long, and Dr. W. O. Nelson. Dr. P. E. Smith was appointed by Dr. Shelton to serve for five years on the Awards Committee.

The general business meeting of the Association was held at 5 o'clock the afternoon of June 9th, the President, Dr. Edgar Allen, presiding. The minutes of the previous meeting, the reports of the Secretary-Treasurer and of the Council were read and accepted. The motion was made and carried that the meeting in 1943 be held the same week and in the same place as that of the Federation of American Societies for Experimental Biology. The report of the election tellers showed that the following officers were elected for the year 1942–43.

President-Elect: Carl Moore

Vice-President: John C. Burch

Secretary-Treasurer: Henry H. Turner

Members of the Council for three years: Edgar Allen, A. T. Kenyon, E. H. Rynearson

Members of the Publication Board for three years: E. M. MacKay, P. E. Smith

Dr. E. Perry McCullagh was appointed Chairman of the Local Committee for the meetings in Cleveland, April 5th and 6th, 1943.

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